PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Yinghui Dan *et al.*

Serial No.: 10/064,001

Filed: June 3, 2002

FOR: A NOVEL METHOD FOR THE PRODUCTION OF TRANSGENIC PLANTS

Group Art Unit: 1638

Examiner: David H. Kruse

Atty. Dkt. No.: 11000023-2229
MONS:130US

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February 19, 2008 /Robert E. Hanson/
Date Robert E. Hanson

BRIEF ON APPEAL

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BRIEF ON APPEAL

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit this Appeal Brief. The date for filing this Brief is February 19, 2008, as February 18, 2008 is a Federal Holiday. The fee for filing this Brief is being concurrently filed. Should any additional fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the enclosed materials, or should an overpayment be made, the Commissioner is authorized to deduct or credit said fees from or to Sonnenschein Nath & Rosenthal LLP, Deposit Account No. 19-3140/11000023-2229/MONS:130US.

I. REAL PARTY IN INTEREST

The Real Party in Interest is Monsanto Company, the parent company of assignee Monsanto Technology LLC.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals.

III. STATUS OF THE CLAIMS

Claims 1-16 were filed with the application. Claims 1-16 were under examination and rejected by the Examiner in the Final Action dated September 18, 2007. Claims 1-16 are therefore the subject of this appeal. A copy of the appealed claims as they currently stand is provided in Section VIII.

IV. STATUS OF AMENDMENTS

Claims 2-16 were amended in the Response to Office Action filed on August 18, 2005 and the amendments were entered by the Examiner. An amendment to claim 1 was made in the Response to Office Action filed on December 11, 2006 and was entered by the Examiner. No subsequent amendments to the claims have been filed.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The subject matter defined in independent claim 1 relates to methods for producing multiple transgenic wheat plants from a single explant by use of a multiple bud inducing medium. Specification at pages 1-2, paragraph [0003]. Independent claim 13 relates to a method for producing a transgenic wheat plant by culturing a wheat mesocotyl explant on a first media comprising thidiazuron to produce buds, introducing exogenous DNA into a cell of a

produced bud, and culturing the bud(s) for production of shoot and plants. Specification at abstract; at claim 13 as originally filed, and at paragraph [0003].

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

(A) Was claim 1 properly rejected under 35 U.S.C. §102(b) as being anticipated by Fry *et al.* (U.S. Patent 5,631,152)?

(B) Were claims 2-16 properly rejected under 35 U.S.C. §103(a) as being obvious over Zhou *et al.* (*Pl. Cell Rep.* 15:159-163, 1995) in view of Tegeder *et al.* (*Pl. Cell Rep.* 15:164-169, 1995), further in view of Weeks *et al.* (*Pl. Physiol.* 102:1007-1084, 1993, still further in view of Cheng *et al.* (*Pl. Physiol.* 115:971-980, 1997)?

(C) Were claims 1-16 properly rejected under 35 U.S.C. §103(a) as being obvious over Fry *et al.* (U.S. Patent 5,631,152) in view of Eudes *et al.* (U.S. Patent 6,995,016)?

(D) Was claim 1 properly rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1 and 7 of U. S. Patent 5,631,152?

VII. ARGUMENT

A. The Claims Are Not Anticipated Under 35 U.S.C. §102(b)

For a reference to anticipate a claim, it must teach every element of the claim such that, "each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987). Because Fry *et al.* do not teach or suggest all elements of Appellants' claimed methods, and specifically use of a multiple bud inducing media in the method for making transgenic wheat according to claim 1, or use of a bud

inducing media with a wheat mesocotyl explant according to claim 13, the anticipation rejection must be reversed.

The Examiner fails to identify where Fry *et al.* explicitly or implicitly teach all of the elements of claim 1. In particular, Fry does not teach the use of a multiple bud-inducing media in a method for making transgenic wheat according to claim 1. For example, the Action states that Fry "disclose a method for producing transgenic wheat comprising culturing explants, introducing exogenous DNA via bombardment, transferring cells from a first media to a second media to induce ***elongation of buds*** into shoots, harvesting and transferring shoots to a culture medium that promotes root development and culturing transferred shoots to produce plants" (Action of July 12, 2007, page 2 last paragraph; emphasis added).

However, claim 1 requires that the explant is cultured in a "***multiple bud inducing*** media suitable for inducing production of a plurality of buds from at least one of said meristems" (emphasis added). As explained in the specification, in a multiple bud inducing media "primary and axillary meristems are induced to generate multiple secondary buds". Specification, paragraph [0003]. These buds may then be cultured in multiple shoot inducing media. Specification, paragraph [0028]. However, a step for producing multiple buds is distinct from a step for elongating any buds, that may be present, into shoots.

Importantly, the present specification describes a bud inducing medium, which may for instance comprise a shoot inducing medium that is supplemented with a cytokinin as well as an auxin. Specification at paragraphs [0034, and 0067-0070]. The term "cytokinin" is not even found in Fry, and the media used in Fry are not disclosed as comprising an added cytokinin. Thus, Fry simply does not describe the element of a "multiple bud inducing" medium within the meaning of claim 1, or a supplemented shoot inducing medium capable of inducing formation of

secondary buds (*i.e.* one supplemented with an added cytokinin; Specification, paragraph [0034], or Table 1). Neither does Fry describe formation of secondary buds. Instead, pre-existing buds are apparently used by Fry, which are then treated to produce shoots (*e.g.* Fry, column 2, lines 27-34; col. 4, lines 27-34).

Additionally, the regeneration of plants from a plurality of buds from multiple explants, as referred to in Fry, is not the equivalent of regeneration of plants from a plurality of buds from a given explant. As noted in the specification, "the primary meristems give rise to multiple secondary buds, in some cases upwards of one hundred secondary buds per primary meristem." Specification at paragraph [0052]. This "allows for an increased output without significantly increasing the amount of labor or plant tissue input." Specification at paragraph [0052]. There is thus no teaching in Fry of a method involving placing the explant on a media suitable for regeneration of multiple buds from a single primary meristem. In sum, Fry does not teach all elements of the claimed invention, and no *prima facie* case for anticipation has been set forth. Reversal of the rejection of claims 1-12 is therefore respectfully requested.

Likewise, the Examiner fails to identify where Fry *et al.* explicitly or implicitly teach all of the elements of claim 13. In particular, the use of a mesocotyl explant is nowhere taught or suggested by Fry. The term "mesocotyl" is simply not found in Fry. As defined in the present Specification, "mesocotyl" refers to "the internode between and including the scutellar node, and the coleoptile". Specification at paragraph [0029]; see also **Exhibit 1**, pages 383-385 for a similar definition in the art. In contrast, Fry uses immature embryo ("IE") and embryogenic callus tissues as explant materials, which do not comprise a mesocotyl as defined. Therefore, Fry does not teach all elements of the claimed invention, and no *prima facie* case for anticipation

of claims 13-16 has been set forth. The anticipation rejection at least of claims 13-16 should therefore be reversed, on this basis.

B. Claims 2-16 Are Nonobvious Under 35 U.S.C. §103(a) Over Zhou *et al.* In View Of Tegeder *et al.*, Weeks *et al.*, And Cheng *et al.*

The Examiner rejects claims 2-16 as being unpatentable over Zhou *et al.* (*Pl. Cell Rep.* 15:159-163, 1995) in view of Tegeder *et al.* (*Pl. Cell Rep.* 15:164-169, 1995), further in view of Weeks *et al.* (*Pl. Physiol.* 102:1007-1084, 1993, still further in view of Cheng *et al.* (*Pl. Physiol.* 115:971-980, 1997). In response, Applicants first note that claim 1 is not included in this obviousness rejection. If claim 1 is understood to be non-obvious, then claims 2-12, which depend on claim 1 and include all of its limitations, must also be non-obvious. Further, the Examiner has failed to identify any reference or combination of references that teaches the use of a multiple bud inducing media, which expressly or inherently comprises a cytokinin, in a method for transforming wheat.

The Examiner alleges that Zhou teaches the culturing of an explant in a bud inducing media (*e.g.* Action of July 12, 2007, page 3, last paragraph). However, Zhou does not describe use of a multiple bud inducing medium, since the ***use of an added cytokinin is not described.*** For instance, at page 160, left column paragraphs 1-3, Zhou describes use of MMS2, CM4, and MMSO media. No cytokinin is stated to be included in any of these media, and the ***production*** of buds is not described. The Action of November 22, 2005 concedes that Zhou *et al.* do not teach the use of cytokinins (Action, page 6, third from last paragraph).

Tegeder is not relevant as outlined below, for instance given that it relates to culture of the dicot *Vicia faba*. Finally, neither Weeks nor Cheng teach are cited as suggesting use of a

cytokinin, nor do they teach or suggest such use. Thus, no *prima facie* case of obviousness has been set forth regarding claims 2-12, and the Action must be reversed.

Further, regarding claims 13-16, Applicants respectfully none of the cited references teach or suggest use of a mesocotyl explant. Zhou describes use of immature embryos, which are non-differentiated, and do not comprise a mesocotyl. Tegeder, apparently added in view of its description of the use of thidiazuron, likewise describes use of protoplasts (and callus derived from protoplasts) from the dicot *V. faba*. However, in contrast to monocots such as Poaceae, which largely grow by stem elongation at or between internodes, dicots do not possess structures termed mesocotyl tissues, since their stems elongate by hypogeal or epigeal development, with elongation occurring at or near the stem apex (**Exhibit 1,** page 385, left column). In any event, the protoplast and callus tissues that are described by Tegeder are not differentiated, and hence cannot comprise a mesocotyl. Weeks, likewise, describes use of embryogenic wheat callus, which does not comprise a mesocotyl. Cheng describes use of wheat tissues from immature embryos, and embryogenic callus. Again, as noted, such explants are not sufficiently differentiated to comprise mescotyl tissue as it is defined in the Specification. Thus, no *prima facie* case of obviousness has been set forth regarding claims 13-16, and the Action must be reversed.

1. The Examiner Has Failed To Demonstrate A Reasonable Expectation Of Success In The Combination Of References Cited By The Action

The Examiner cites Tegeder *et al.,* apparently regarding use of thidiazuron. This reference describes treatment of protoplasts and calli from the leguminous dicot *Vicia faba* (Fava bean) with thidiazuron, a cytokinin. However, this citation is not relevant. First, one of skill in the art of plant cell culture would not routinely apply conditions for culture of *V. faba*, which is a dicotyledonous plant, to tissue culture of wheat, a highly diverged monocotyledonous plant.

Thus, there would be no reasonable expectation of success in applying Tegeder to wheat, given the known differences in culture conditions for these two unrelated species of plants. The vast differences in culture responses between monocots and dicots have previously been analyzed (*e.g. Plant Genetic Systems N.V. v. DeKalb Genetics Corp.,* Fed. Cir., No. 02-1011, 1/13/03; **Exhibit 2**.).

Applicants also note that the response of the *V. faba* cells to different media conditions as described by Tegeder was clearly distinct from the responses of wheat cells described in the present specification. For instance, Tegeder states that only one cultivar responded to their treatment (Tegeder, page 166 right column 3rd and 4th paragraphs; Table 3; and page 168, right column, first paragraph of "Discussion"). Given that *V. faba* is not closely related to wheat, and that only one bean cultivar tested by Tegeder even showed any shoot-forming response, one of skill in the art of plant regeneration would have held no expectation that any given cultivar of wheat, a greatly diverged monocot species, would respond as well, let alone in a genotype independent manner. Further, Tegeder states that many months (at least 10 months; Tegeder, page 166, right column) was required to proceed through their bean culture process. However, it is known in the art that culture of wheat cells for such extended periods of time as was required for *V. faba* would be detrimental to the ability of wheat cells to successfully regenerate fertile plants. This is described in the cited reference by Weeks **(Exhibit 3)** and references mentioned therein. For instance, "...regenerability of such cultures declines with time..." (*e.g.* page 1077, right column). Thus, barring impermissible hindsight, a skilled worker would have had no expectation of success in routinely applying the teachings of Tegeder *et al.* to wheat culture to obtain fertile transformed wheat plants.

2. The Claimed Invention Yields Unexpected Results

As explained above, the claimed method yields production of **multiple** secondary buds. See Specification, paragraph [0052]. These secondary buds may then be used as a target for transformation, thus increasing the number of target cells per explant, allowing for an increased output of transformed material without significantly increasing the amount of labor or plant tissue input. These results are significant in that they represent a potential increase of orders of magnitude in the number of transformed wheat plants obtained, on a per explant basis. In contrast, the cited Fry reference states, at Table 2, that use of the "rapid" technique resulted in a transformation frequency of bout 1.3%. That is, of 2675 embryos subjected to transformation, 36 glyphosate tolerant plants were recovered. In contrast to Fry *et al.*,, Table 5 of the Specification shows that the present method can result in 49-93 buds (and hence potentially transformed plants) **per explant**, while Table 6 shows that even with cv. Autry, the least efficient cultivar in Table 6, more than 60% of explants could produce more than 20-80 buds per primary meristem in an explant, again demonstrating a significant improvement in the ability to efficiently regenerate wheat plants following transformation procedures. Table 8 shows that 20-48 elongated shoots could be produced, again **per explant**. Tables 11 and 13 likewise demonstrate that up to 10-30% of explants could produce stably transformed glyphosate tolerant plants, again at least an order of magnitude improvement over the wheat transformation described in the art. The present method thus allows a substantial and unexpected increase in the efficiency of the transformation process.

3. The Cited References Are Not Properly Combinable

In addition to failing to show that all of the elements of the instant claims can be found or derived from the prior art, there would be no motivation or suggestion to combine the cited

references to arrive at the present invention. The only motivation to combine the references cited by the Examiner is that "Weeks *et al* teach that wheat is the largest crop in the world in terms of production and that monocotyledonous plants, cereals in particular, have lagged behind dicotyledonous plants in ease and efficiency of transformation," (page 7 of the Action dated November 22, 2005). However, Applicants fail to see how this provides any motivation to combine, for example, a wheat transformation method of Zhou with a cytokinin media of Tegeder. The Examiner in multiple Office Actions fails to clarify what motivation is to be found, merely repeating the unfounded assertion that the cited references are "reasonably pertinent" and that therefore such a combination would have been obvious. Furthermore, Applicants note that, while Zhou concerns methods for transforming wheat, Tegeder concerns methods for regenerating an unrelated plant, *Vicia faba*. Thus, these references constitute non-analogous prior art and are not properly combinable. Hence, a motivation is lacking to combine the cited references to produce the claimed method for transforming wheat.

The cited references also do not describe genotype independent transformation and regeneration of wheat, and when Tegeder is combined with the other references, the references, in total, teach away from any expectation that such an effect could be achieved. Further, in Tegeder, rooting was not even achieved, and the described method requiring 10-14 months of culture, would in total teach away from any attempt to apply the method for efficiently producing fertile transgenic wheat plants.

The Action of September 18, 2007 further states that none of the cited references teach that multiple transgenic plants cannot be produced from a single explant. Applicants respectfully submit that the cited references are pertinent only insofar as to what they actually teach, rather

than what they do not teach cannot be done. It is unclear how to even rebut such an argument, and Applicants urge the removal of a rejection made on such a basis.

In view of the foregoing, reversal of the obviousness rejection is respectfully requested.

C. Claims 1-16 Are Nonobvious Under 35 U.S.C. §103(a) Over Fry *et al.* in view of Eudes *et al.* (U.S. Patent 6,995,016).

The Examiner asserts that Eudes (**Exhibit 4;** column 4, lines 41-54) teaches that the hormone content of a plant tissue culture medium is of great importance. While this may be true, it a teaching of only the most general type, and the Examiner's conclusion that bud inducing media would therefore comprise cytokinins and auxins does not flow from this teaching in any necessary or logical manner. Simply put, Eudes does not suggest how the present result might be achieved. A close analysis instead shows that, if anything, Eudes teaches away from the presently claimed subject matter, and the present results are unexpected in view of Eudes even when combined with Fry.

Applicants respectfully reiterate, regarding claims 1-16, that Fry does not teach use of a bud inducing media, or of cytokinin to form multiple buds in wheat explants. Eudes is apparently added in view of its alleged teachings regarding uses of hormones in plant cell culture. However, Eudes teaches use of ***immature scutella*** cells from embryos or callus through direct or indirect embryogenesis, *e.g.* "monocotyledonous plant cells are cultured under conditions conducive to direct formation of primary embryos without an intervening callus stage." (Eudes, col. 7, lines 11-14, although a callus stage may also be utilized, but still requiring embryogenesis; see also Eudes col. 8, lines 34-49). However, although Eudes mentions an organogenic approach in the Background of Invention section (Eudes, column 3, line 23 and following), organogenesis is explicitly described as comprising "...the development

of...(o)nly one meristem..." (Eudes, col. 3, lines 29-31). Eudes also describes an organogenic approach in their Detailed Description (*e.g.* col. 15, line 59 and following). However, this approach requires prior embryogenesis. Further, Eudes does not suggest ***wheat*** in this context. Thus, Eudes apparently ***does not recognize that multiple meristems or additional buds may be formed in the presence of a cytokinin***, in wheat. Eudes is therefore not properly applied, and the presently described results would be unexpected over Fry in view of Eudes.

Additionally, as a result, Eudes teaches that embryogenesis is preferred over organogenesis. (*e.g.* Eudes, col. 3, lines 48-62). This clearly teaches away from the present invention, which instead relates to manipulation of explants comprising meristems without the disadvantages of an embryogenic approach which were known to include, for instance, genotype dependence, somaclonal variation, poor regeneration, and/or reduced fertility. Specification, *e.g.* paragraph [0028]. If anything, a skilled worker would conclude, after reading Eudes, that the presently claimed approach would ***not*** be expected to yield efficient methods for producing transgenic wheat plants, *i.e.* there would be no reasonable expectation of success in combining the teachings of Fry and Eudes in pursuing a non-embryogenic approach for obtaining transgenic wheat plants.

Further regarding claims 13-16, Applicants respectfully note, as outlined above, that Fry does not teach or suggest use of mesocotyl tissue, and the addition of Eudes does not cure this defect. Mesocotyl tissue is in no way taught or suggested by Eudes, which instead describes an embryogenic approach starting from embryo or callus tissues.

In view of the foregoing, reversal of the obviousness rejection is respectfully requested.

D. Obviousness Type Double Patenting

The Action has failed to set forth a *prima facie* case for obviousness type double patenting regarding claim 1. The instant rejection, first stated in the Office Action dated November 22, 2005, and subsequently repeated, merely states that claim 1 is "not patentability distinct" from claims 1 and 7 of Fry *et al.* (U.S. Patent 5,631,152). However, the Action fails to set forth a case for obviousness of the instant claims over Fry in combination with any of the other references of record in the case. Specifically, Applicants fully demonstrate herein above that Fry *et al.* fails to teach or suggest all elements of the claims alone or in combination with the remaining cited art. The rejection is therefore without support.

Further, the Action states that the steps of the cited method of Fry are "similar" to those of the present application. Applicants respectfully note, as outlined above, that Fry does not teach the use of multiple bud inducing media, *e.g.* comprising added cytokinin. Thus the presently claimed steps are not "similar", but are clearly distinct from those of Fry, and reversal of the rejection on this basis is respectfully requested.

CONCLUSION

It is respectfully submitted, in light of the above, that none of the claims are properly rejected. Therefore, Appellants respectfully request that the Board reverse the pending grounds for rejection.

Respectfully submitted,

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Date: February 19, 2008

VIII. CLAIMS APPENDIX

APPEALED CLAIMS:

1. (Previously presented) A method of producing multiple transgenic wheat plants from a single explant comprising:

providing an explant presenting a plurality of meristems;

culturing said explant in a first multiple bud inducing media suitable for inducing production of a plurality of buds from at least one of said meristems;

introducing exogenous DNA into more than one of said plurality of buds;

removing said plurality of buds from said first media and transferring said plurality of buds to a second media suitable for induction of elongation of said buds into shoots;

harvesting and transferring said shoots to a culture medium that promotes root development; and

culturing said transferred shoots to produce multiple transgenic wheat plants.

2. (Previously presented) The method of claims 1, wherein said multiple bud inducing media comprises a cytokinin and an auxin.

3. (Previously presented) The method of claims 2, wherein said cytokinin is thidiazuron.

4. (Previously presented) The method of claims 2, wherein the concentration of said cytokinin is between 2.0mg/L and 7.5mg/L.

5. (Previously presented) The method of claims 2, wherein said cytokinin is thidiazuron and said auxin is selected from the group consisting of 2,4-D and picloram.

6. (Previously presented) The method of claims 5, wherein the concentration of thidiazuron is between 2.0mg/L and 7.5mg/L and the concentration of auxin is between 0.5mg/L and 2.0mg/L.

7. (Previously presented) The method of claims 1, wherein said plurality of meristems contains the scutellar node.

8. (Previously presented) The method of claims 1, wherein said explant is a wheat mesocotyl explant.

9. (Previously presented) The method of claims 1, wherein said exogenous DNA comprises a nucleic acid encoding a protein capable of conferring resistance to a selection agent.

10. (Previously presented) The method of claims 9, further comprising a step of selecting for plants containing the protein conferring resistance to a selection agent.

11. (Previously presented) The method of claims 1, wherein said exogenous DNA is introduced via biolistic particle bombardment.

12. (Previously presented) The method of claims 1, wherein said exogenous DNA is introduced via *Agrobacterium*-mediated transformation.

13. (Previously presented) A method of producing multiple transgenic wheat plants from a single explant comprising:

providing a wheat mesocotyl explant presenting a plurality of meristems;

culturing said wheat mesocotyl explant on a first media, comprising thidiazuron at a concentration of between about 2.0 mg/L and 7.5 mg/L, and 2,4-D at a concentration of about 0.5 mg/L and 2.0 mg/L, to induce the production of a plurality of buds from at least one of said plurality of meristems;

introducing exogenous DNA into at least one of the cells of said plurality of buds;

removing said plurality of buds from said first media and transferring said plurality of buds to a second media suitable for induction of elongation of said buds into shoots;

culturing said shoots to produce multiple transgenic wheat plants.

14. (Previously presented) The method of claim 13, wherein said exogenous DNA is introduced via *Agrobacterium*-mediated transformation.

15. (Previously presented) The method of claim 13, wherein said exogenous DNA is introduced via biolistic particle bombardment.

16. (Previously presented) The method of claim 13, further comprising a step of selecting for plants containing the exogenous DNA.

IX. EVIDENCE APPENDIX

Exhibit 1: Salisbury & Ross, *Plant Physiology*, 3rd Edition. Wadsworth Publishing, Belmont, California, 1986, pp. 383-385.

Exhibit 2: *Plant Genetic Systems N.V. v. DeKalb Genetics Corp.,* Fed. Cir., No. 02-1011, 1/13/03.

Exhibit 3: Weeks *et al., Pl. Physiol.* 102:1077-1084, 1993.

Exhibit 4: Eudes *et al.,* U. S. Patent 6,995,016.

X. RELATED PROCEEDINGS APPENDIX

None.

EXHIBIT 1

PLANT PHYSIOLOGY

THIRD EDITION

Frank B. Salisbury
Utah State University

Cleon W. Ross
Colorado State University

Wadsworth Publishing Company
Belmont, California
A Division of Wadsworth, Inc.

kept dark or given far-red (Nabors and Lang, ...bar conclusion is that P_{fr} increases the growth ...of the radicle cells, presumably those in the elon... region, by decreasing their water potential so that ...re easily absorb water from soils and germinate.

These facts suggest that germination of light-...ng seeds fails in darkness because the radicle ...t grow with sufficient force to break through ...vers that surround it. Of these layers, the let-...radicle is restricted almost entirely by the tough ...perm, even though it is only two or three cell ...thick. The endosperm is also the restrictive ...in *Phacelia tanacetifolia* and various *Syringa* (lilac) ...s. For lettuce, only increased thrust of the rad-...eems important, even though the endosperm ...r is weakened greatly after germination is well ...way (Bewley and Black, 1982). For other seeds, ...ight reasonably expect P_{fr} to increase germi-...n either by increasing radicle thrust or by weak-...g surrounding barriers to its growth, or both. ...dormancy is less a mystery when we consider ...nation as a struggle between the growth poten-...t the radicle and the growth-restrictive mechan-...ffects of surrounding layers. In some cases, the ...al restriction is great; in others it is of little ...quence, and only a small increase in the radicle ...th potential caused by P_{fr} is enough to cause ...nation. Nevertheless, even in these, the re-...l of P_{fr} by far-red light reduces germination.

...ls of Hormones on Photodormancy In most ...dormant seeds, applied gibberellins substitute ...e light requirement; and for a few species such ...tuce, cytokinins also substitute for light or par-...replace it. Auxins usually do not promote ger-...ion of photodormant or nondormant seeds and ...stead either innocuous at low concentrations or ...tory at high concentrations. The role of eth-...is less clear. It cannot break photodormancy, ...can partially overcome other kinds of seed ...ncy in cocklebur and in certain peanut and ...varieties. It can also partially overcome dor-...caused by high temperatures in lettuce and ...photodormancy problems in cocklebur ...hi et al., 1983). Abscisic acid almost always ...germination, because of its growth inhibitory ...

...llectively, these results suggest that P_{fr} might ...photodormancy by causing synthesis of a gib-...n or a cytokinin or by destroying an inhibitor ...s ABA. The evidence about this is presently ...versial (Bewley and Black, 1982; DeGreef and ...icq, 1983), but no one has yet measured hor-...changes only in the radicle or hypocotyl cells ...e responsible for germination. This seems es-...to understand relations among light, growth ...ers, and growth inhibitors in photodormancy

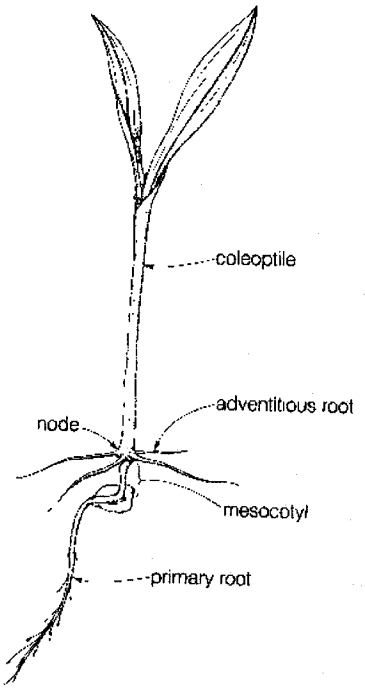

Figure 19-7 Some morphological characteristics of a week-old maize seedling grown in light. The coleoptile has stopped elongating, and two leaves have broken through it and have largely unrolled. The shoot apex is at the node where adventitious (prop) roots originate. The mesocotyl is the first internode formed above the seed storage tissues and the scutellum (cotyledon) in the seed.

and other kinds of seed dormancy discussed in Chapter 21. Analyses of whole seeds for hormone levels seem nearly useless in understanding hormonal aspects of dormancy, because the whole seed is so large relative to the few tissues that control germination.

19.5 The Role of Light in Seedling Establishment and Later Vegetative Growth

Once germination is accomplished, further plant development still remains subject to control by light. We introduced some of these controls in Section 19.1 and Fig. 19-1. We now evaluate these and other effects and ask whether phytochrome is the only pigment involved and how it acts.

Development of Poaceae Seedlings After a grass or cereal grain seed germinates, its coleoptile elongates until the tip breaks through the soil. Between the scutellum (see Fig. 16-12) and the base of the coleoptile is an internode called the **mesocotyl** (first internode, Fig. 19-7) that in some species elongates greatly

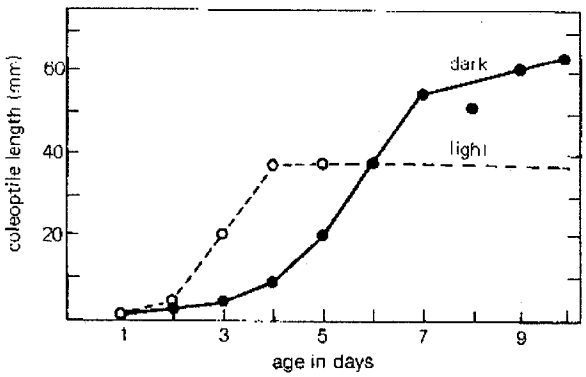

Figure 19-8 Elongation of oat coleoptiles in darkness and in continuous white light. Light at first promotes growth but later is inhibitory. (From B. Thomson, 1954.)

after germination of deeply planted seeds. Elongation of the mesocotyl, coleoptile, and leaves enclosed by the coleoptile is necessary to carry leaves into the light and to establish near the soil surface the adventitious roots produced at the node just above the mesocotyl (Fig. 19-7). Elongation of the mesocotyl has received attention for more than 40 years. All results show that mesocotyl elongation is extremely sensitive to light (Mandoli and Briggs, 1981; Shäfer et al., 1982). In oats, growth of the mesocotyl is slowed over a 54-hour period by continuous red light at photon irradiance levels of only 5×10^{-16} moles of photons per square meter of tissue per second (Schafer et al., 1982). This irradiance level is only about 10^{-7} that in all visible wavelengths provided by moonlight. This effect of light on plants is by far the most sensitive one known, and no green safelights are safe in studies with this response.

Elongation of the coleoptile must equal or exceed that of the leaves it encloses as they grow upward together; otherwise the leaves would grow out of the coleoptile and probably be broken off in the soil. Growth rates of these two organs are coordinated until they reach the soil surface and are exposed to light. After exposure to light, the leaves become green and photosynthetic, and they break through the coleoptile tip. Leaf emergence occurs because light promotes leaf elongation and decreases the extent to which coleoptiles can elongate (although it speeds their early elongation) (Schopfer et al., 1982). Furthermore, breakage of the coleoptile tip by the elongating first leaf stops coleoptile elongation, presumably because this stops auxin transport from the tip to elongating cells below. The light promotion of leaf growth and inhibition of coleoptile growth are phytochrome responses of sunlight. Figure 19-8 shows that the overall effect of continuous light is to reduce coleoptile elongation, even though during the first few days elongation is promoted. The reasonable conclusion from these 1954 results is that light has-

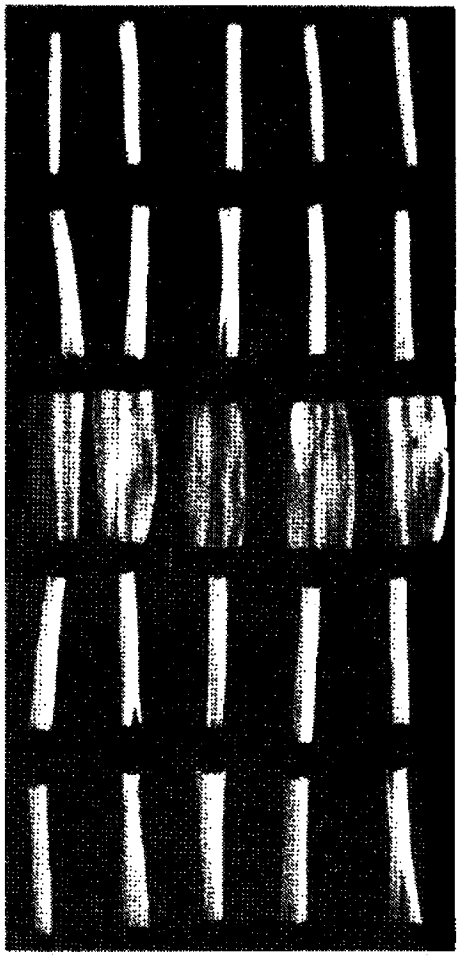

Figure 19-9 Effect of pretreatment with red and far-red on unrolling of leaf sections from etiolated maize seedlings. Red promotes opening, whereas subsequent far-red treatment nullifies the red effect. (From W. H. Klein, L. Price and K. Mitrakos, 1963.)

tens growth and maturation of young coleoptiles because those cells begin to elongate earlier and mature earlier than in darkness. Effects of light on elongating cells reaching maturity show that elongation is slowed and that maturation occurs earlier than in darkness. More recent results verify those conclusions and further show that phytochrome is the controlling photoreceptor of light, even though long-term light exposure that results in high irradiance values is necessary to cause the responses (Schäfer et al., 1982).

Figure 19-7 illustrates a maize seedling grown in light from a seed planted near the soil surface. The mesocotyl had elongated very little, and the first two leaves had emerged from the coleoptile. Each of these leaves was rolled up inside the coleoptile, but when exposed to light they began to unroll (flatten). Rolling was still evident only at the point

parture from the broken coleoptile. Unrolling of grass leaves is controlled by a typical phytochrome response, low irradiance levels of red promoting and subsequent far-red nullifying the red effect (Fig. 19-9). Low energies of far-red are without effect, and low energy blue is only slightly promotive except in rice. Unrolling is caused by more rapid growth of cells on the concave (to be uppermost) than the convex side. Whether this growth is caused by wall loosening, solute production that decreases the cells' osmotic potential, or both, is not known. Nevertheless, exogenous gibberellins and, to a lesser extent, cytokinins replace the need for light and P_{fr} (DeGreef and Fredericq, 1983). These results suggest that P_{fr} causes rolled leaves to form gibberellins or cytokinins which then cause unrolling. This hypothesis might be correct for gibberellins, because P_{fr} promotes gibberellin production and release from young plastids in rolled wheat and barley leaves much sooner than the leaves unroll. No studies showing light effects on cytokinin contents of unrolling leaves are available, so for now it seems safest to conclude that light might induce leaf unrolling by causing production of gibberellins in concave cells. Alternatively, the concave cells might become more sensitive to the hormone levels they already contain when exposed to light (see the personal essay by A. Trewavas in Chapter 16).

Development of Dicot Seedlings In dicots, the cotyledons either remain underground by **hypogeal development,** as in pea, or emerge above ground **epigeally,** as in beans, radish, and lettuce. In either case, a hook is formed near the stem apex that pushes up through the soil and pulls with it the fragile young leaves or cotyledons. (In Fig. 19-1 this hook has moved, as it does in seedlings that develop epigeally, into the epicotyl (stem section above cotyledons) and has opened somewhat, perhaps by slight light exposure during watering.) As mentioned in Section 15.2, this hook forms as a result of unequal growth on the two sides of the hypocotyl or epicotyl in response to ethylene soon after germination. As the hook emerges from the soil, red light acting through P_{fr} promotes opening of the hook. Hook opening apparently results from inhibition by light of ethylene synthesis in the hook. Differential growth that results from faster elongation of cells on the lower (concave) side than on the upper (convex) side causes hook opening (Section 15.3). Accompanying this, light increases leaf blade expansion, petiole elongation, chlorophyll formation, and chloroplast development, as in grass leaves (Fig. 19-1), and P_{fr} also speeds petiole elongation.

Most of the light promotion of leaf growth, at least in dicots, is caused by an HIR (Dale, 1982). A good example is provided by the primary leaves of bean. Plants grown 10 days under dim red light have slightly larger leaves and substantially more cells than those kept in darkness; but when they are transferred to white light, cell expansion and leaf growth increase greatly. In this case the HIR system causes expansion by enhancing acidification of the cell walls, thus loosening them so that they grow faster under turgor pressure (Van Volkenburgh and Cleland, 1981).

Light effects on chlorophyll formation and chloroplast development result first from a triggering action of P_{fr} that causes production of **delta-aminolevulinic acid (ALA),** probably from glutamic acid (Kasemir, 1983; Castelfranco and Beale, 1983). ALA is the metabolic precursor converted into each of the four pyrrole rings of chlorophyll. Nevertheless, ALA is not converted all the way to chlorophyll without higher irradiance red or blue light. Instead, the metabolic pathway stops when a compound often called **protochlorophyll** is formed. More accurately, protochlorophyll is **protochlorophyllide *a,*** which differs from chlorophyll *a* (Fig. 9-4) only by the absence of a phytol tail and two H atoms. Protochlorophyllide *a* is rapidly reduced to chlorophyllide *a* in red or blue light, because protochlorophyllide *a*, like chlorophylls, absorbs those photons effectively. Addition of the phytol tail, an isoprenoid formed from the mevalonic acid pathway (Chapter 14), completes formation of chlorophyll *a*; some of the chlorophyll *a* is then converted to chlorophyll *b*. Chloroplast development depends strongly on chlorophyll formation and, therefore, on both of these light effects, although there are other light responses that we shall not discuss (Kasemir, 1983; Virgin and Egnéus, 1983). All of these responses lead, within a few hours, to photosynthesis in grass leaves as they break through the coleoptile and in cotyledons or young leaves of dicots as they break through the soil. Cotyledons of conifers somehow form chlorophyll and become photosynthetic even in darkness, but their needles require light for these processes.

As photosynthesis begins in leaves and cotyledons, stem elongation is inhibited by light. Of course, the seedling cannot elongate after its food supplies are exhausted; but while carbohydrates or fats are still plentiful, light is inhibitory. This inhibition of stem elongation was apparently first recorded by Julius von Sachs in 1852. He observed that stems of many species do not grow as fast during daylight as they do at night. We now realize that blue, red, and far-red all contribute to this phenomenon and that cryptochrome and phytochrome are both responsible. Hans Mohr and others in Germany worked extensively with etiolated seedlings of white mustard *(Sinapis alba)* and measured many responses in these, which are summarized in Table 19-1. They also measured the action spectrum for inhibition of

EXHIBIT 2

United States Court of Appeals for the Federal Circuit

02-1011

PLANT GENETIC SYSTEMS, N.V.
(now known as Aventis CropScience N.V.),

Plaintiff-Appellant,

and

BIOGEN, INC.,

Plaintiff,

v.

DEKALB GENETICS CORPORATION,

Defendant-Appellee.

Eric H. Weisblatt, Burns, Doane, Swecker & Mathis, L.L.P., of Alexandria, Virginia, argued for plaintiff-appellant. With him on the brief were Susan J. Dadio, R. Danny Huntington, and Barbara Webb Walker.

John F. Lynch, Howrey Simon Arnold & White, LLP, of Houston, Texas, argued for defendant-appellee. With him on the brief were Thomas A. Miller, and Richard L. Stanley. Of counsel on the brief Daniel T. Shvodian, Howrey Simon Arnold & White, LLP, of Menlo Park, California. Of counsel was Hemant H. Kewalramani, Howrey Simon Arnold & White, LLP, of Houston, Texas.

Appealed from: United States District Court for the District of Connecticut

Judge Dominic J. Squatrito

United States Court of Appeals for the Federal Circuit

02-1011

PLANT GENETIC SYSTEMS, N.V.
(now known as Aventis CropScience N.V.),

Plaintiff-Appellant,

and

BIOGEN, INC.

Plaintiff,

v.

DEKALB GENETICS CORPORATION,

Defendant-Appellee.

DECIDED: January 13, 2003

Before NEWMAN, MICHEL, and PROST, Circuit Judges.
MICHEL, Circuit Judge.

Plant Genetic Systems, N.V. ("PGS") appeals the September 7, 2001 decision of the United States District Court for the District of Connecticut, Plant Genetic Sys., N.V. v. DeKalb Genetics Corp., 175 F. Supp. 2d 246 (D. Conn. 2001), entering judgment for the accused infringer, DeKalb Genetics Corporation ("DeKalb"), on all counts of the complaint in this patent infringement suit. The case was tried to the bench for thirteen days. At the end of the trial, the district court concluded that claims 1-5 and 10-11 of PGS' patent, U. S. Pat. No. 5,561,236 ("the '236 patent"), were invalid for lack of enablement and that claims 8-9 and 12-15, the remaining asserted claims, were not infringed. Id. at 270. Because as to enablement, the defendant established a need for undue experimentation and as to non-infringement, that the properly construed claims did not cover the defendant's products, we affirm.

BACKGROUND

This case involves only one patent, the '236 patent, which claims a priority date of March 11, 1987. The patent issued on October 1, 1996 to inventors from PGS and Biogen Corporation and is directed to transgenic plant cells, plants, and seeds. DeKalb makes and sells transgenic corn seeds. PGS sued DeKalb for patent infringement the day the '236 patent issued.

The '236 patent and DeKalb's transgenic corn products relate to the herbicide-resistant characteristics of a plant or plant cell. Non-selective herbicides may indiscriminately kill most plants by blocking an essential biochemical process of these plants -- metabolizing ammonia via the action of glutamine synthetase. Thus, these herbicides are also called glutamine synthetase inhibitors. Two structurally-related compounds, bialaphos and glufosinate, are such herbicides. It has been desirable to obtain food plants that are resistant to non-selective herbicides. Herbicide-resistant plants can grow in the presence of the herbicide that kills other unwanted plants or weeds.

The '236 patent teaches a plant cell, or a plant or seed containing such a cell, that is genetically engineered so that the cell can produce a protein that prevents herbicides such as bialaphos or glufosinate from blocking the function of glutamine synthetase. The mechanism of the engineering is to incorporate into the genome of the plant cell a gene (today called either the bar gene or the pat gene)[1], the protein product of which can inactivate a glutamine synthetase inhibitor.

The claims in issue are claims 1-5 and 8-15. Claims 1-5 and 10-11 include the cell, tissue and culture claims and are referred to, post, as "the cell claims." This group of claims all depends on claim 1, which reads:

> A plant cell having a heterologous DNA stably integrated into its genome; said DNA comprising a heterologous DNA fragment encoding a protein having an acetyl transferase activity which inactivates a glutamine synthetase inhibitor in said cell.

Claims 8-9 and 12-15 are referred to by the parties and herein as the "plant and seed claims." Claim 8 is representative of this group of claims and reads:

> A plant which consists of the cells of claim 1 and which is susceptible to

> infection and transformation by Agrobacterium and capable of regeneration thereafter.

A key issue of this case is the scope of both sets of the claims, i.e., what kind of plants or plant cells are covered by the claims. Flowering plants can be broadly categorized as monocotyledons ("monocots") and dicotyledons ("dicots"), depending on whether the initial development of the seed produces one leaf (monocot) or two leaves (dicot). In the '236 patent, all the working examples are dicots, for example, tomato, potato, and tobacco plants. However, the accused infringing product made and sold by DeKalb is corn -- a monocot. Additionally, the scientific community was not able to transform monocots until after it first transformed dicots. Thus, it is disputed whether the cell claims of the '236 patent, which are agreed by the parties literally to cover all plant cells, were enabled for monocots on March 11, 1987. It is also disputed whether the plant and seed claims are correctly interpreted to exclude monocots, therefore leaving DeKalb's corn products outside the scope of these claims.

The district court, at its thirteen-day bench trial, heard evidence on various transformation techniques and the chronology of scientific progress regarding monocot transformation. Plant Genetic Sys., 175 F. Supp. 2d at 255-64. At the end, the court found that despite the teachings of the specification, practicing stable gene transformation for monocot cells in 1987 required undue experimentation and, thus, the cell claims of the '236 patent were proven invalid for lack of enablement. Id. at 265.

The district court also construed the limitation "susceptible to infection and transformation by Agrobacterium and capable of regeneration" that was added to the plant and seed claims during prosecution to overcome the Examiner's rejection for non-enablement. The court held that a person skilled in the art at the time the parent application was filed would have understood the plant and seed claims to exclude monocots. Id. at 266-68. The court additionally considered trial testimony that no methodology existed as of the effective filing date of the '236 patent by which monocots could be infected and transformed by Agrobacterium to produce plants capable of regeneration. Id. at 268-69. The court viewed this testimony as further supporting its claim construction. Thus, the court held that DeKalb's

transgenic corn products could not infringe the plant and seed claims because corn is indisputably a monocot. Id. at 269-70.

Both the invalidity and non-infringement holdings have been appealed.

DISCUSSION

Enablement is ultimately a question of law which this court reviews de novo. Durel Corp. v. Osram Sylvania Inc., 256 F.3d 1298, 1307 (Fed. Cir. 2001). However, the factual findings underlying the legal conclusion are reviewed for clear error. PPG Indus., Inc. v. Guardian Indus. Corp., 75 F.3d 1558, 1564 (Fed. Cir. 1996). Claim constructions are reviewed de novo. Cybor Corp. v. FAS Techs., 138 F.3d 1448, 1454-55 (Fed. Cir. 1998) (en banc).

I

PGS asks this court to reverse the district court's invalidity decision regarding claims 1-5 and 10-11 because, PGS asserts, the district court (A) failed to make any findings regarding the pioneering nature of the invention claimed in the '236 patent; (B) improperly shifted the burden of proof to the patentee to establish enablement; (C) failed to consider all of the relevant evidence; and (D) misused post-effective filing date work in its enablement determination. After considering each of PGS' arguments, for the reasons set forth below we conclude that the district court did not clearly err in its evidentiary findings and did not err in its conclusion that DeKalb had proven non-enablement by clear and convincing evidence.

A

PGS argues that the '236 patent is a pioneer patent and thereby entitled to a broad scope of coverage and lower standard of enablement. PGS asserts that, in failing to make any findings regarding the pioneering status of the invention of the '236 patent, the district court committed reversible error.

A patent application is required to "contain a written description of the invention, and of the manner and process of making and using it . . . as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same." 35 U.S.C. § 112, ¶ 1 (2000). "To be enabling, the specification of the patent must teach those

skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" Genentech Inc. v. Novo Nordisk A/S, 108 F.3d 1361, 1365 (Fed. Cir. 1997) (quoting In re Wright, 999 F.2d 1557, 1561 (Fed. Cir. 1993)). Enablement is determined as of the effective filing date of the patent, In re Hogan, 559 F.2d 595, 604 (CCPA 1977), which, it is undisputed, is March 11, 1987.

We reject appellant's argument that the district court erred for making no finding on whether the claimed invention was "pioneering." Such a finding was unnecessary to the disposition of this case. We have previously affirmed a lack-of-enablement rejection by the United States Patent and Trademark Office ("PTO") without addressing the "pioneering" issue. In re Vaeck, 947 F.2d 488, 495-96 (Fed. Cir. 1991). Even in Hogan, on which PGS heavily relies, the Court of Customs and Patent Appeals stated, in addressing the Board of Patent Appeals' failure to consider the "pioneer" status of appellants' invention, that "[w]hether appellants' invention is of 'pioneer' status is not before us and bears no relation to our decision herein, though such status may influence the decision required on remand." Hogan, 559 F.2d at 602 n.10. Thus, we hold that the district court's failure here to make findings on the "pioneer" status of the '236 patent, without more, was not erroneous.

Further, even assuming the court had made such a finding, and properly, PGS' argument that the '236 patent is thereby entitled to a lower enablement requirement is not supported by precedents. The first paragraph of 35 U.S.C. § 112 effectively requires that "the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art." In re Fisher, 427 F.2d 833, 839 (CCPA 1970) (holding that the appellant, who was the first to achieve a potency of greater than 1.0 for adrenocorticotrophic hormones ("ACTHs"), had not enabled the preparation of ACTHs having potencies much greater than 2.3, and the claim recitations of potency of "at least 1" rendered the claims insufficiently supported under the first paragraph of 35 U.S.C. § 112). To determine whether there is a reasonable correlation between the scope of the claims and the scope of enablement, the degree of predictability of the relevant art may need to be considered. Id.; Vaeck, 947 F.2d at 495.

PGS notes that Fisher also stated that "such an inventor should be allowed to dominate the future patentable inventions of others where those inventions were based in some way on his teachings." Fisher, 427 F.2d at 839. This dictum, however, only sets the context for Fisher's holding that "[i]t is equally apparent, however, that [the inventor] must not be permitted to achieve this dominance by claims which are insufficiently supported and hence not in compliance with the first paragraph of 35 USC 112." Id.

PGS also argues that the courts should consider the effect of Hogan on the principles set forth by Fisher. However, we do not agree with PGS that Hogan alters the principles set forth in Fisher and commands an outcome favorable to PGS. In Hogan, a patent application, having a priority date in 1953, claimed a solid polymer of propylene. Although the claims were not limited to the crystalline form of the polymer but also encompassed amorphous forms, amorphous propylene did not exist until 1962. Hogan, 559 F.2d at 605. The methods disclosed in the specification were not able to produce amorphous polymers that later became possible to produce. Id. On appeal from a Board of Patent Appeals decision that the application was not enabling as to how to prepare amorphous propylene, the Court of Customs and Patent Appeals held that the PTO erred in relying on the later state of the art in assessing enablement. Id. at 605-07. The court remanded the enablement issue for consideration of whether the application was enabling in view of the state of the art existing in 1953. Id. at 609.

We do not read Hogan as allowing an inventor to claim what was specifically desired but difficult to obtain at the time the application was filed, unless the patent discloses how to make and use it. In Hogan, amorphous propylene, on the record before the court, was not known or in existence when the application was filed. In the present case, however, monocots existed in 1987 and stably-transformed monocot cells were highly desirable. PGS indeed asserts that monocot cells were already being stably transformed. Thus, monocots and stably transformed monocot cells were not an unknown concept that came into existence only after 1987. But stably transformed monocot cells were difficult to produce, and the '236 patent gave no instruction how.

Moreover, Hogan cannot be read to assist "improper enforcement against later

developers." Id. at 607. Hogan simply held that one could not use a later-existing state of the art to invalidate a patent that was enabled for what it claimed at the time of filing. In addressing the issue of whether a claim may be of sufficient breadth to cover the later state of the art, Hogan stated:

> The business of the PTO is patentability, not infringement. Like the judicially-developed doctrine of equivalents, designed to protect the patentee with respect to later-developed variations of the claimed invention, the judicially-developed "reverse doctrine of equivalents," requiring interpretation of claims in light of the specification, may be safely relied upon to preclude improper enforcement against later developers.

Id. If the present case were comparable to Hogan, PGS could avoid invalidation of the cell claims by at least asserting that these claims were not understood by those skilled in the art as encompassing monocots when the '236 patent was filed. However, PGS concedes that the cell claims cover monocot cells. Only by doing so can PGS sue DeKalb, which makes monocot products, for infringement. Having agreed that the cell claims encompass monocot cells, a later development, PGS' reliance on Hogan ignores the validity-infringement differentiation Hogan made.

Regarding PGS' extensive citation of statements from Hogan such as that pioneering inventions "deserve broad claims to the broad concept," id. at 606, we conclude that they are taken out of context and thus unconvincing. As the concurrence in Hogan pointed out, these statements are "extended dicta." Id. at 610. We do not need to address all of the insightful comments made by the concurring judge; it is sufficient for the present case that we hold the district court did not err in not applying Hogan's dicta to its enablement analysis.

PGS also cites Hormone Research Foundation Inc. v. Genentech, Inc., 904 F.2d 1558, 1568 (Fed. Cir. 1990), for its proposition that "a rigid application of the enablement requirement cannot be permitted to destroy the incentives in our patent system that encourage the early disclosure of pioneering inventions." In Hormone Research, the enablement challenge focused on the lack of disclosure regarding how to make the compound as later produced with higher purity and potency. This court vacated a summary judgment of non-enablement because, inter alia, it was not clear from the record whether the technology of making the

compound of higher purity and potency existed at the time the application was filed, and therefore, "[f]urther factual developments as to the state of the art at the date of the application . . . [were] required." Id. at 1568-69.

Again, PGS relies on dicta from Hormone Research but ignores the holding of the case. In both Hogan and Hormone Research, which relied on Hogan, compounds having better qualities did not seem to be in existence on the date when the patent applications were filed, but the claims (albeit with a narrower scope) might be nevertheless enabled in view of the state of the art then existing. In the present case, PGS does not allege that monocots or stably transformed monocot cells were not in existence in 1987 or that the cell claims were enabled in 1987 under the standard enablement analysis. Instead, PGS attempts to use the dicta from Hogan and Hormone Research to expand the coverage of claims, yet create a new, lower standard of enablement.

We conclude that the law does not support PGS' assertion that the '236 patent is entitled to both a broad scope of coverage and a lower standard of enablement. The extended dicta PGS cites cannot be used to alter the holdings of these precedents. PGS' reliance on Hogan and related cases is misplaced. DeKalb distinguishes these cases from the present one on the ground that each of these cases involves a method to make the claimed invention as of the filing date and a later-developed state of the art pertaining to an improved version of the patented invention, i.e., having better properties than originally possible, while the '236 patent did not disclose any method of achieving transgenic monocots and, therefore, the later development with monocots was not merely an improvement. In view of our analysis as stated above, we need not treat DeKalb's argument.

B

PGS next argues that the district court improperly shifted a burden to prove enablement to it when the burden of proving non-enablement properly rests on the accused infringer. We do not agree that the district court did so. The district court, at the outset of its opinion, properly stated that "all United States patents enjoy a presumption of validity" and that "the burden of proving that the '236 patent is not enabled rests squarely with DeKalb." Plant

Genetic Sys., 175 F. Supp. 2d at 253-54. Furthermore, the district court specified that DeKalb "bears the burden of presenting clear and convincing evidence that the cell claims were not enabled as of March 11, 1987, the effective filing date of the 236 patent." Id. at 254. After analyzing various cell transformation techniques available in 1987, the district court concluded that "the defendant has shown by clear and convincing evidence that the cell claims of the '236 patent were not enabled." Id. at 265.

PGS asserts that the district court shifted the burden of proof to PGS because it improperly used In re Goodman, 11 F.3d 1046 (Fed. Cir. 1993). In analyzing the Agrobacterium-mediated transformation method, the district court used, as a starting point, Goodman, which held that Goodman's patent claims covering all "plant cells," filed in 1985, were not enabled because the record showed that no reliable Agrobacterium-mediated transformation method for use with monocots existed in 1985. Plant Genetic Sys., 175 F. Supp. 2d at 256-57. The court then proceeded to determine whether, on the evidence in this case, reliable Agrobacterium-mediated transformation methods developed for monocots between 1985 and 1987. Id. at 258-62. PGS believes that, by adopting findings in Goodman for the state of art in 1985 and then looking for evidence between 1985 and 1987 to alter the Goodman conclusion, the district court shifted DeKalb's burden to prove non-enablement to PGS' burden to show enablement.

We conclude that the district court did not shift the burden of proof to PGS by using Goodman as a starting point. There is no assertion that the district court excluded evidence that could rebut the findings of Goodman. Thus, Goodman was not used as an "irrebuttable factual presumption[]," as PGS asserts. The district court would have improperly used Goodman if the court used Goodman to show the state of art in 1985 regardless of evidence showing results contrary to Goodman. This was not the case.

Neither did the district court shift the burden of proof to PGS when it looked for any evidence between 1985 and 1987 that could alter Goodman's findings on Agrobacterium-mediated monocot transformation. The district court's search for such evidence of enablement merely indicated the strength of the evidence of non-enablement, rather than a shift of the

burden to prove enablement to PGS. We hold that the district court did not commit legal error in using Goodman as a starting point and then looking for any subsequent evidence, before it concluded that DeKalb proved non-enablement by clear and convincing evidence.

C

PGS argues that the district court failed to consider all of the relevant evidence before reaching its conclusion of non-enablement. PGS asserts that the district court committed legal error by not considering a 1987 abstract by Goldman, Graves, and Roberts, a 1988 publication of Rhodes et al. that referred to a 1986 work of Fromm, and three patents, U.S. Patent Nos. 5,177,010, 5,187,073, and 6,020,539,[2] awarded for Goldman and Graves' 1986 method of transforming corn seedlings.

The fact that the district court did not in its opinion recite every piece of evidence does not mean that the evidence was not considered. FMC Corp. v. Hennessy Indus., Inc., 836 F.2d 521, 524 (Fed. Cir. 1987). "We presume that a fact finder reviews all the evidence presented unless he explicitly expresses otherwise." Medtronic, Inc. v. Daig Corp., 789 F.2d 903, 906 (Fed. Cir. 1986). "'The ultimate test of the adequacy of findings is whether they are sufficiently comprehensive and pertinent to the issue to form a basis for the decision (and whether they are supported by the evidence).'" Id. (quoting Loctite Corp. v. Ultraseal Ltd., 781 F.2d 861, 873 (Fed. Cir. 1985)).

No evidence shows that the district court did not consider the documents that PGS cites. According to DeKalb, all these allegedly ignored documents were presented at trial, but found unpersuasive in view of the evidence of non-enablement. The district court indeed heard numerous pieces of evidence in the thirteen-day trial and issued a thorough decision discussing various transformation techniques at issue. See Plant Genetic Sys., 175 F. Supp. 2d at 250-65. The court found that, in view of the publication itself and the testimony from other researchers, Goldman and Graves' 1986 publication did not enable one to transform corn cells using Agrobacterium without undue experimentation. Id. at 258-62. The court made the same finding regarding Fromm's electroporation method published in 1986. Id. at 262-63.

We thus do not agree with PGS that the district court ignored the 1987 abstract, the

three patents, and the publication of Rhodes et al. These documents relate to the 1986 work of Goldman and Graves or that of Fromm. The district court, after weighing all evidence, apparently found that the 1986 work of Goldman and Graves and that of Fromm did not enable one skilled in the art to stably transform corn cells without undue experimentation. The district court did not commit legal error for not reciting the documents that referred to or originated from the publications of Goldman and Graves and Fromm. Neither did it clearly err in finding that certain pieces of evidence had more weight than others or were more worth discussing.

Having held that the district court did not clearly err in its evidentiary findings, it is not necessary for us to address DeKalb's arguments that (1) the data published in the 1987 abstract by Goldman et al. were characterized by an expert as "extremely questionable," (2) PGS' assertion regarding the abstract conflicts with its later request for research funding to determine the feasibility of maize transformation by A. tumefaciens, and (3) the presumed validity of the three patents allegedly based on Goldman and Graves' 1986 work cannot be used as irrebuttable proof that one skilled in the art was able, in 1987, to practice the invention of the cell claims of the '236 patent, and that the district court considered evidence not available to the PTO in evaluating the weight of these three patents.

D

PGS further argues that the district court misused post-filing-date work in its non-enablement determination. PGS states that the district court erred in using work relevant to a later-existing state of the art to test whether the cell claims were enabled in 1987.

We do not agree with PGS' characterization of the district court's analysis. The district court indeed cited post-1987 reports indicating first transformation of corn cells. However, as Hogan stated:

> This court has approved use of later publications as evidence of the state of art existing on the filing date of an application. That approval does not extend, however, to the use of a later . . . publication disclosing a later (1962) existing state of the art in testing an earlier (1953) application for compliance with § 112, first paragraph. The difference may be described as that between the permissible application of later knowledge about art-related facts existing on the filing date and the impermissible application of later knowledge about later art-related facts . . . which did not exist on the filing date.

Hogan, 559 F.2d at 605. Clearly, the district court looked into post-1987 reports to determine whether monocot cells were readily transformable in 1987 rather than to show that monocot cells could be successfully transformed in 1990. Report of a first success after 1987 indicates failure or difficulty in or before 1987. Thus, the district court properly used later reports as evidence of the state of the art existing in 1987.

We have considered PGS' other arguments regarding enablement of the cell claims, including its reliance on an unpublished district court decision, [3] and find them unpersuasive. [4]

To the contrary, we conclude that the district court properly conducted its enablement analysis under In re Wands, 858 F.2d 731 (Fed. Cir. 1988), before reaching its conclusion that stable transformation of monocot cells required undue experimentation on March 11, 1987. The district court did not commit the legal error asserted by PGS. Nor did the court clearly err in its evidentiary findings. We therefore uphold the district court's conclusion that the cell claims are invalid because they were proven by clear and convincing evidence not to have been enabled by the specification of the '236 patent in view of the then-state of the art.

II

PGS asks this court to reject the district court's claim construction for the plant and seed claims and, therefore, reverse its non-infringement decision.

The district court construed the added limitation "susceptible to infection and transformation by Agrobacterium and capable of regeneration" of the plant and seed claims to mean that, at the time the patent application was originally filed, a person skilled in the art would understand the claim limitation to exclude monocots. Plant Genetic Sys., 175 F. Supp. 2d at 266-68. The specification of the '236 patent does not disclose any method or working examples for so transforming monocot plants or cells. However, the district court found this not to be determinative in excluding monocots from the claim coverage. On the other hand, the court found that, during the prosecution of the patent application, the patentees disclaimed monocots when they added the limitation of "susceptible to infection and transformation by Agrobacterium and capable of regeneration" to overcome the examiner's non-enablement

rejection, which was based on the fact that "there [was] no evidence that fertile transgenic plants [could] be regenerated in most agronomic monocots, as in the case of maize or rice." Id. at 267-68 (citing the Examiner's Office Action dated April 17, 1989) (emphasis added). Finally, the court considered trial testimony asserting that no methodology existed in 1987 that could transform monocots with Agrobacterium to produce plants capable of regeneration. It accepted that testimony. The district court held that the plant and seed claims did not cover monocots such as corn ("maize"), and accordingly, DeKalb's transgenic corn products could not infringe the plant and seed claims.

A

PGS argues that the district court ignored the plain meaning of the claim language. As written, claims 8-9 and 12-15 literally cover any plant or seed that consists of the cells of claim 1 and is "susceptible to infection and transformation by Agrobacterium and capable of regeneration." PGS asserts that the lack of the word "dicot" in these claims indicates that these claims are not so limited.

We hold that the district court did not ignore the plain meaning of the claims, but properly gave objective meaning to them as they were understood at the time the patent application was filed. Claims are to be given their ordinary and objective meaning as of the time of the invention. Kopykake Enters., Inc. v. Lucks Co., 264 F.3d 1377, 1383 (Fed. Cir. 2001). "[W]hen a claim term understood to have a narrow meaning when the application is filed later acquires a broader definition, the literal scope of the term is limited to what it was understood to mean at the time of filing." Id. (citation omitted).

Although we agree that lack of any disclosure on monocot transformation in the specification is not determinative, the prosecution history of the '236 patent shed light on the meaning of the plant and seed claims as they were understood at the time of filing. During prosecution of the patent application, the patentees added the limitation "susceptible to infection and transformation by Agrobacterium and capable of regeneration" to overcome the Examiner's rejection of non-enablement as to monocots, including "maize" (corn). Before the limitation was added, the Examiner cited the lack of evidence on monocot transformation and regeneration and the "undue amount of experimentation [required] to set forth the conditions necessary for plant cell transformation and/or plant regeneration for the different plants as broadly claimed [as] in the instant application." The Examiner's Office Action, April 17, 1989. We conclude that the plant and seed claims were only allowed because the limitation on transformation and regeneration was added. Thus, patentees amended the plant and seed

claims effectively to exclude monocots from the claims. This conclusion is inescapable even though neither the word "dicots" appears in the claims as a positive limitation, nor the word "monocots" as a limitation of exclusion. Therefore, based on prosecution history alone, the claims at issue cannot cover corn or any other monocots.

B

PGS also argues that the district court erred in considering extrinsic evidence to construe claims 8-9 and 12-15. We disagree. PGS' assertion that the district court "improperly relied upon extrinsic evidence . . . to change the plain and ordinary meaning of the claim" is without merit.

To properly construe claims, a court must always examine the claims, the rest of the specification, and, if in evidence, the prosecution history. Vitronics Corp. v. Conceptronic, Inc., 90 F.3d 1576, 1582 (Fed. Cir. 1996). However, Vitronics does not bar the courts from ever considering extrinsic evidence. Pitney Bowes, Inc. v. Hewlett Packard Co., 182 F.3d 1298, 1308 (Fed. Cir. 1999). In fact, "it is entirely appropriate, perhaps even preferable, for a court to consult trustworthy extrinsic evidence to ensure that the claim construction . . . is not inconsistent with clearly expressed, plainly apposite and widely held understandings in the pertinent technical field." Id.

We hold that the district court did not abuse its discretion in admitting or otherwise err in considering or relying on such extrinsic evidence. The court first examined intrinsic evidence such as the specification and the prosecution history, which, it determined, indicated that addition of the limitation on transformation by Agrobacterium and regeneration was intended and understood to exclude monocots. Plant Genetic Sys., 175 F. Supp. 2d at 267-68. Having so determined, the district court consulted extrinsic evidence to ensure that its interpretation of the claim language was not inconsistent with the understanding in the technical field as of the filing date of the patent. Such use of extrinsic evidence is permitted.

We thus conclude that the district court did not err in its construction that claims 8-9 and 12-15 covered only dicots. Because DeKalb's allegedly infringing products are unquestionably monocots, the district court properly found that the plant and seed claims were not infringed. At the least, its finding of non-infringement cannot be viewed as clearly erroneous.

CONCLUSION

We hold that the district court did not err in concluding that claims 1-5 and 10-11 were proven by defendant to be invalid for lack of enablement. We also hold that the district court correctly construed claims 8-9 and 12-15 to exclude monocots and therefore could not have erred, much less clearly erred, in holding that these claims were not infringed. Accordingly, the judgment of the district court is, in all respects challenged on appeal,

AFFIRMED.

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[1] The *bar* gene is also widely used as a selectable marker, whose herbicide resistance character can help determine whether other genes linked to it have also been incorporated into a cell.

[2] These three patents issued in 1993 and 2000 from either divisional or continuation-in-part applications that were based on one abandoned application filed on June 30, 1986.

[3] Chiron Corp. v. Abbott Labs., No. C-93-4380, 1996 WL 209717 (N.D. Cal. Apr. 23, 1996).

[4] At the oral argument on appeal, PGS also stated that the district court erred by applying evidence of difficulties in monocot plant transformation to monocot cells. We conclude that the district court did not clearly err in its evidentiary findings. Regarding enablement of the cell claims, although the court used the words "corn transformation" or "transform corn" in its analysis of post-1985 developments in Agrobacterium-mediated transformation, it clearly was addressing the issue of corn-cell transformation rather than transformation and regeneration of a whole corn plant. Plant Genetic Sys., 175 F. Supp. 2d at 258-62. Additionally, the court's analysis of electroporation and microprojectile bombardment as well as its conclusion drawn to the cell claims unambiguously focused on corn cells rather than plants.

EXHIBIT 3

Plant Physiol. (1993) 102: 1077–1084

Rapid Production of Multiple Independent Lines of Fertile Transgenic Wheat (*Triticum aestivum*)[1]

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Improvement of wheat (*Triticum aestivum*) by biotechnological approaches is currently limited by a lack of efficient and reliable transformation methodology. In this report, we detail a protocol for transformation of a highly embryogenic wheat cultivar, Bobwhite. Calli derived from immature embryos, 0.5 to 1 mm long, were bombarded with microprojectiles coated with DNA containing as marker genes the *bar* gene, encoding phosphinothricin-resistance, and the gene encoding β-glucuronidase (GUS), each under control of a maize ubiquitin promoter. The bombardment was performed 5 d after embryo excision, just after initiation of callus proliferation. The ability of plantlets to root in the presence of 1 or 3 mg/L of bialaphos was the most reliable selection criteria used to identify transformed plants. Stable transformation was confirmed by marker gene expression assays and the presence of the *bar* sequences in high molecular weight chromosomal DNA of the resultant plants. Nine independent lines of fertile transgenic wheat plants have been obtained thus far, at a frequency of 1 to 2 per 1000 embryos bombarded. On average, 168 d elapsed between embryo excision for bombardment and anthesis of the T_0 plants. The transmission of both the resistance phenotype and *bar* DNA to the T_1 generation verified that germline transformation had occurred.

Many of the recent advances in plant science have resulted from application of the analytical power of recombinant DNA technology coupled with plant transformation. These approaches facilitate studies of the effects of specific gene alterations on plant development and physiology. They also make possible the direct manipulation of genes to bioengineer improved plant varieties.

Monocotyledonous plants, and cereal crops in particular, have lagged behind dicotyledonous plants in ease and efficiency of transformation. Rice was the first major cereal crop transformed. Toriyama et al. (1988), Zhang and Wu (1988), and Shimamoto et al. (1989) all used direct DNA delivery into regeneration-competent protoplasts to obtain transgenic plants. Although this approach continues to be the main procedure for rice transformation (Peng et al., 1992), an alternative was developed by Christou et al. (1991), who used immature embryos as the target tissue and electric discharge particle acceleration as the DNA delivery method.

Because it is difficult to regenerate fertile plants from protoplasts of cereals other than rice, the initial production of fertile transgenic maize (Fromm et al., 1990; Gordon-Kamm et al., 1990), oat (Somers et al., 1992), and sugarcane (Bower and Birch, 1992) plants used embryogenic suspension cell or callus cultures as target tissues and microprojectile bombardment as the mechanism of DNA delivery. In all these cases, the ability to regenerate plants depended on the establishment of long-term embryogenic cell cultures. Recently, it has been shown that the establishment of such cultures is not necessary for successful transformation of maize: D'Halluin et al. (1992) used wounded immature embryos as target tissues and electroporation for DNA delivery to produce transformed plants.

Although wheat (*Triticum aestivum*) is the largest crop in the world in terms of production, it was the last among economically important cereals to be transformed. Vasil et al. (1991) produced stably transformed wheat suspension-cell cultures from which they were unable to regenerate plants. More recently, Vasil et al. (1992) obtained several transformed callus lines after microprojectile bombardment of embryogenic callus and selection with the herbicide Basta. From one of these lines, transformed wheat plants were regenerated. These plants were unable to self-fertilize, but progeny could be produced by outcrossing to either wild-type pollen or ova. These T_1 progeny were fully fertile and transmitted the transformed phenotype to at least the T_2 generation.

Although the work of Vasil and collaborators is a landmark in efforts to develop wheat transformation, the protocol they report is limited in its utility by its dependence on the identification and establishment of a specific callus type in long-term tissue cultures. Establishment of the regenerability of such cultures requires several months, is limited to certain genotypes, and declines with time (Redway et al., 1990; Vasil et al., 1992). To circumvent these limitations, we reasoned that a more suitable target for wheat transformation might be the callus produced by immature embryos shortly after excision and culturing. Immature embryos of the hexaploid wheat cultivar Bobwhite form such callus tissue, which can be maintained and regenerated into fertile plants with high

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Abbreviations: *bar*, the sequences of the *bar* gene from *Streptomyces hygroscopicus* that encode phosphinothricin acetyl transferase and confer resistance to bialaphos and Basta; GUS, β-glucuronidase, encoded by the *uidA* gene from *Escherichia coli*; MS medium, Murashige and Skoog medium; PAT, phosphinothricin acetyl transferase.

frequency (T. Weeks, unpublished data; J. Driver, A. Guenzi, and T. Peeper, unpublished data). We now report use of this tissue as the target for DNA delivery by microprojectile bombardment and a protocol for wheat transformation that has resulted in the production of multiple independent lines of fertile transgenic plants.

MATERIALS AND METHODS

Callus Culture and Plant Growth Conditions

Wheat plants (*Triticum aestivum* L. em. Thell. cv Bobwhite) were grown in a greenhouse in Albany, CA. To establish callus cultures, caryopses 10 to 18 d postanthesis were surface-sterilized with 70% ethanol for 5 min and 20% sodium hypochlorite for 15 min, followed by two changes of sterile distilled water. Immature embryos, 0.5 to 1 mm long, were aseptically removed under a stereo dissecting microscope and placed with the scutella exposed on MS medium (Murashige and Skoog, 1962) modified for wheat cell culture (Sears and Deckard, 1982) and solidified with 0.25% (w/v) Phytagel (Sigma Chemical Co.[2]). Calli cultures were maintained at 27°C with a 16-h photoperiod (43 $\mu E/m^2$) on MS medium with 2% Suc and 1.5 mg/L of 2,4-D and transferred to new medium at 2-week intervals. For regeneration, embryogenic calli were transferred to MS medium with 0.5 mg/L dicamba (Sandoz Crop Protection, Des Plaines, IL) as described by Hunsinger and Schauz (1987). Calli-derived shoots were transferred to Pyrex culture test tubes (25 × 150 mm) containing rooting media composed of half-strength MS without hormones. For selection after bombardment, agar media at each stage were supplemented with 1 mg/L of bialaphos (Meiji Seika Kasha, Tokyo, Japan).

Plantlets were transferred from rooting media to pots of Sunshine soil mixture No. 1 (Fisons Horticulture Co., Mississauga, Canada) and acclimated to lower humidity at 21°C with a 16-h photoperiod (300 $\mu E/m^2$) in an environmental chamber. After 2 weeks, plants were transferred to the greenhouse. These primary regenerants are called T_0 plants. The first generation progeny of these plants are called T_1 plants. These were obtained by excising embryos from T_0 plants 15 DAF and germinating them on MS medium solidified with 0.25% Phytagel agar.

Plasmid DNA

Plasmid pAHC25, the vector used for wheat transformation, was kindly provided by Alan Christensen and Peter Quail (Plant Gene Expression Center, University of California Berkeley/U.S. Department of Agriculture, Albany, CA). This dual-expression vector consists of the *uidA* (Jefferson et al., 1987) and *bar* (Thompson et al., 1987) genes, each under control of the maize ubiquitin *Ubi1* promoter (Christensen et al., 1992; A.H. Christensen and P.H. Quail, unpublished data). The *bar* gene encodes the enzyme PAT, which inactivates phosphinothricin, the active ingredient of the herbicides bialaphos and Basta. The *uidA* gene encodes the enzyme GUS. Plasmid DNA was purified from alkaline-lysed cells on CsCl gradients and stored at a concentration of 1 mg/mL in Tris-EDTA buffer, pH 8.0 (Sambrook et al., 1989).

Microprojectile Bombardment

Prior to bombardment, 1-μm gold particles were coated with pAHC25 DNA by the procedure of Daines (1990). A stock suspension of gold particles (Bio-Rad) was suspended at 60 mg/mL in absolute ethanol. Thirty-five microliters of the suspension were aliquoted into 1.5-mL microcentrifuge tubes, washed in sterile distilled water, and resuspended in 25 μL of Tris-EDTA containing 25 μg of supercoiled plasmid DNA. The following solutions were added in order: 220 μL of sterile water, 250 μL of 2.5 M $CaCl_2$, and 50 μL of 0.1 M spermidine (free base). The microcentrifuge tubes were shaken with a vortex mixer at 4°C for 10 min and centrifuged at 16,000*g* for 5 min. The supernatant was removed, and the pellet was washed with 600 μL of ethanol. The DNA-coated gold pellets were resuspended in 36 μL of ethanol. For bombardment, 10 μL of the DNA-gold suspension was placed in the center of a macroprojectile.

Approximately 25 embryos were placed in the center of a 15 × 100 mm Petri dish containing callus maintenance medium solidified with 0.35% Phytagel. After 5 d in culture, the embryo-derived calli were bombarded under vacuum with pAHC25-coated gold particles, using the helium-driven DuPont Biolistic Delivery System (model PDS-1000) and disposable components supplied by Bio-Rad. The distance from the stopping plate to the target was 13 cm, and the rupture disc strength was 1100 p.s.i. Immediately after bombardment, calli were transferred to MS selection media containing 1 mg/L of bialaphos.

Enzyme Assays

GUS activity (Jefferson et al., 1987) was assessed histochemically using the same buffer used by Perl et al. (1992) except that the substrate 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid was purchased from Jersey Lab and Glove Supply (Livingston, NJ). PAT activity was determined in extracts prepared from 8-cm sections of leaf tips 1 week after regenerated plants were transferred to soil. The acetylation assay was performed as described by Spencer et al. (1990) except that a Wheaton hand grinder was used to prepare the extracts instead of a bead beater, and 2.5 μL instead of 3 μL of [^{14}C]acetyl-CoA (55.9 mCi/mmol, New England Nuclear-Dupont) were used as label. The products of the reaction corresponding to 2.4 μg of total protein were loaded into each lane of a Whatman (Maidstone, England) LHP-KDF high-performance TLC plate.

Wheat DNA Isolation and Gel Blot Analysis

Wheat genomic DNA was isolated as described by D'Ovidio et al. (1992) and quantitated by measuring A_{260}. Fifty micrograms of each DNA sample were digested with *Hin*dIII in 300 μL of the manufacturer's (GIBCO BRL) buffer overnight. Twenty-five micrograms of digested or undigested DNA were separated by electrophoresis through 0.6% aga-

[2] The use of a brand name by the U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

rose (FMC Corp., Rockland, ME) gels in Tris-borate-EDTA (Sambrook et al., 1989) buffer. To reconstruct a single copy of plasmid per wheat hexaploid genome, 25 μg of *Hin*dIII-digested nontransformed wheat DNA was mixed with 1.6 pg of a 3.8-kb *Pvu*I/*Xho*I fragment of pAHC25 that includes the *bar* coding region.

For blot analysis, DNA was transferred (Southern, 1975) to a Nytran membrane (Schleicher & Schuell). DNA was fixed to the membrane by UV cross-linking, using a Stratalinker (Stratagene). Prehybridizations were carried out at 65°C for 3 h in a variant of the solution used by Devey et al. (1991): 4× SSPE (150 mM NaCl, 10 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM EDTA, pH 7.7), 10× Denhardt's reagent, 1% (w/v) SDS, 1% (w/v) nonfat dry milk, and 100 μg/mL of denatured salmon sperm DNA. Hybridizations were carried out for 40 h at 65°C in 4× SSPE, 5× Denhardt's solution, 0.5% (w/v) SDS, 1% (w/v) nonfat dry milk, 50 μg/mL of denatured salmon sperm DNA, 10% (w/v) dextran sulfate, and 50 × 10^6 cpm of ^{32}P-radiolabeled probe. The probe consisted of the *bar* coding region isolated as a 570-bp *Pst*I fragment from pAHC25 and labeled with [^{32}P]dCTP (New England Nuclear) using the Random Primed DNA Labeling Kit supplied by Boehringer Mannheim Biochemicals. After hybridization, membranes were rinsed with 1× SSPE, 0.2% SDS at room temperature, and washed twice for 45 min each at 65°C in 0.25× SSPE, 0.2% SDS. Damp filters were exposed at −80°C on Kodak XAR-5 film with an intensifying screen.

Herbicide Application

Basta TX (Hoechst AG, Frankfurt am Mein, Germany) is a commercial formulation of phosphinothricin (200 g/L). A 2% solution of the herbicide was sprayed on greenhouse-grown plants, which were then observed over a 3-week period.

RESULTS

Initiation, Bombardment, and Selection of Callus Tissue

The wheat cv Bobwhite was chosen for use in transformation experiments because of its high frequency of regeneration from tissue culture to fertile plants (J. Driver, A. Guenzi, and T. Peeper, unpublished data). The steps of the transformation process are shown in Figure 1. Immature embryos 0.5 to 1 mm in length were excised from greenhouse-grown plants (10–18 d after anthesis, depending on the time of year) and placed, scutellum side exposed, on callus maintenance media containing 1.5 mg/L of 2,4-D (Fig. 2A). Five days after initiation into tissue culture, proliferating callus tissue is visible at the edges of the embryos (Fig. 2B). At this stage, the embryos were bombarded with gold particles coated with 7 μg of the plasmid pAHC25, which contains the *bar* and GUS marker genes, each under control of a maize ubiquitin promoter (Christensen et al., 1992). The first experiment utilized older callus tissue and transient GUS expression to establish efficient DNA bombardment conditions. Figure 2C shows the pattern of ubiquitin:GUS gene expression in a piece of 30-d-old embryogenic callus visualized with the histochemical assay 2 d after bombardment. Discrete, intensely blue sectors are seen distributed over the entire surface. In the center of the target tissue, the staining appears confluent.

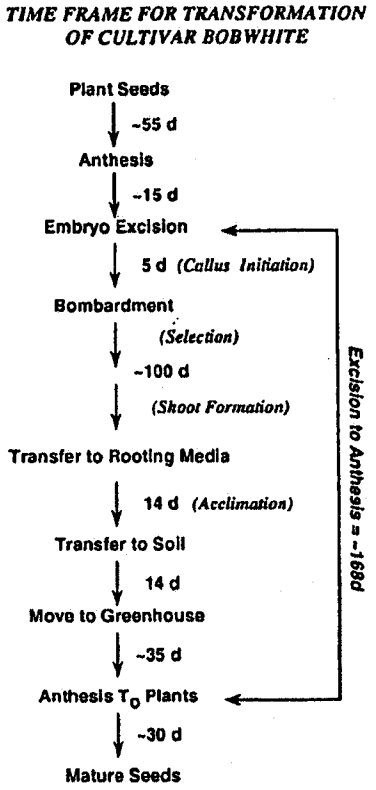

Figure 1. Time frame for events and processes in the production of transgenic Bobwhite plants. The times shown are averages for experiments performed in 1992. The times designated by ~ are approximate and vary with either the season of the year in which the plants are grown or the individual callus line or plant.

In subsequent transformation experiments, these bombardment conditions were used to deliver pAHC25 DNA to embryos 5 d after excision and culturing. Immediately after bombardment, the embryos were transferred to callus maintenance media containing 1 mg/L of bialaphos for selection of resistant tissues. In preliminary experiments, this level of bialaphos had inhibited growth of normal callus 27% in 3 weeks and 67% in 6 weeks (data not shown). Healthy, growing sectors of callus were transferred every other week to fresh selection media. By 6 to 7 weeks, resistant callus tissue could be clearly identified, and individual cell lines were established. These lines are designated in this report by their bombardment number followed by an alphabetical letter. When callus pieces from some of the lines were tested for GUS gene expression by the histochemical assay, the resistant callus displayed uniformly dark-blue staining (Fig. 2D), indicative of the presence and expression of the ubiquitin:GUS portion of the vector. Control callus remained yellowish-white in the presence of the substrate (not shown).

Resistant callus lines were maintained on selection media. At variable times after bombardment, green sectors, the precursors of shoots, originated on portions of the callus (Fig. 2E). At this stage, callus pieces were divided and the greening portions were transferred to regeneration media (MS with

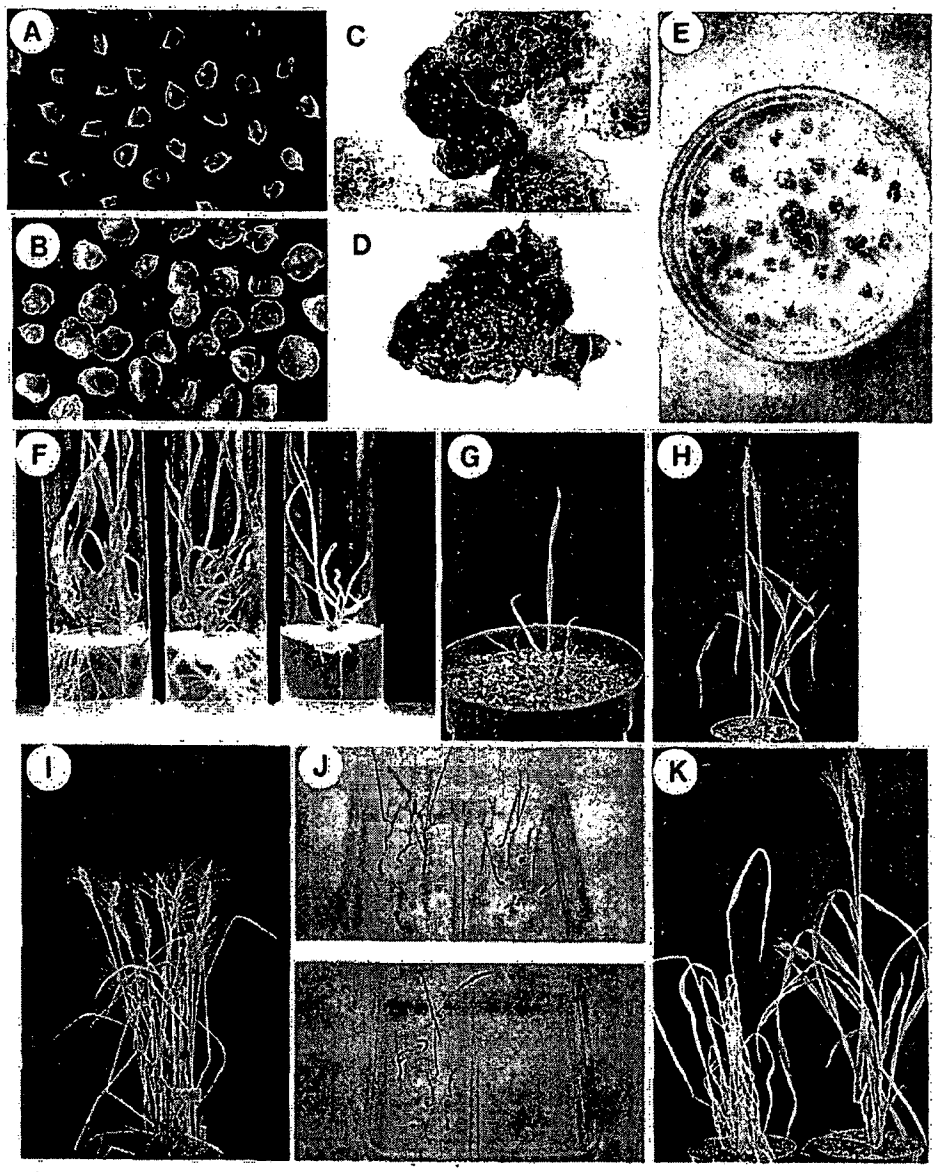

Figure 2. Wheat callus and plant tissues. A, Immature Bobwhite embryos excised 15 d postanthesis on agar medium. B, Proliferation of callus tissue from embryos after 5 d on MS medium. C, Wheat callus tissue 30 d after culturing from immature embryos, stained histochemically for GUS activity 2 d after bombardment with pAHC25. D, Bialaphos-resistant tissue undergoing shoot (protrusion) regeneration, stained histochemically for GUS activity. E, Callus tissue on MS media containing 1 mg/L of bialaphos 70 d after bombardment. F, Regeneration of roots; Left, Nontransformed plantlet in rooting medium lacking bialaphos; center, putative transformant in rooting medium supplemented with 3 mg/L of bialaphos; right, nontransformed plantlet in rooting medium with 3 mg/L of bialaphos. G, Transformed plant from line 12M shortly after transfer to the greenhouse. H, Transformed plant from line 12K at the heading stage (grown during the winter months). I, Transformed plant from line 9C at maturity (grown during the summer months). J, Selection of T_1 progeny on germination media. Immature embryos from a T_0 plant of line 2A (left of each panel) and a nontransformed control plant (right of each panel) excised 15 DAF and germinated on MS agar media with (lower) and without (upper) 3 mg/L of bialaphos. Seedlings were photographed after 18 d. K, Selection of T_1 progeny at the boot stage. A T_1 plant from line 2A (right) and a nontransformed plant (left) photographed 2 weeks after spraying with 2% Basta.

0.5 mg/L of dicamba). In most cases, sufficient callus was present underlying the differentiating tissues to maintain the callus line on selection media. Usually, multiple plants could be regenerated from each line.

Differentiated shoots were transferred to culture tubes containing rooting media (half-strength MS) with 1 or 3 mg/L of bialaphos. Only about 10% of the shoots that had been able to organize on the regeneration media were able to form roots in media containing either level of bialaphos (Fig. 2F). The plantlets were scored as resistant at this stage by their ability to form long, highly branched roots in the bialaphos-containing medium (center in Fig. 2F), similar to the roots produced by nontransformed plantlets in medium lacking the herbicide (left in Fig. 2F). Sensitive plantlets (right in Fig. 2F) initiated root formation, but the primary root soon stopped growing and put out only a few short lateral roots. Aerial portions of sensitive plantlets exhibited yellow necrosis and reduced vigor within 1 week, whereas resistant plantlets thrived in the rooting media.

After 2 weeks, plantlets established under bialaphos selection were transferred to soil. In a growth chamber under high humidity, they were allowed to acclimate to greenhouse conditions for 2 weeks. Upon transfer to the greenhouse, the young plants exhibited the curled and spindly appearance (Fig. 2G) typical of nontransformed Bobwhite plants regenerated from tissue culture. As regenerated plants matured and flowered, they regained a normal appearance (Fig. 2, H and I). Some of the lines had normal levels of fertility and seed set. However, most had reduced seed set compared with nontransformed Bobwhite plants regenerated from tissue culture. Only one line, 6K, was completely sterile.

Figure 1 summarizes the transformation and regeneration process and shows average times required for each of the steps. Of the total of 168 d between excision of the target embryos and anthesis of regenerated plants, about 119 d of the process are spent in tissue culture. Exact times varied for each regenerant, depending on how quickly resistant callus lines were established and how quickly shoots developed.

DNA Analysis

DNA was extracted from leaf tissue of T_0 plants derived from independent callus lines and analyzed for the presence of *bar* DNA (Fig. 3). DNA from nontransformed plants (lanes labeled NT) exhibit no hybridization to the *bar* coding region fragment used as probe. Lanes containing uncut DNA from plants 2A, 9S, and 6K show hybridization to a DNA band of high mol wt. This result is indicative of integration of *bar* sequences into wheat chromosomal DNA.

*Hin*dIII-digested DNAs from six independent transformation events are shown in panels A, B, and C of Figure 3. DNA from line 2A is included in each panel so that the relative intensities of the bands can be compared between the different blots. As expected for independent events, each plant had a unique integration pattern of DNA fragments. Two events obtained from bombardment 9, but selected as independent callus lines, have different patterns (lanes 9G and 9S). The patterns ranged from simple to complex. DNA from line 2A contains only two bands with homology to the *bar* probe, each present at about one copy per hexaploid genome as judged by comparison of their intensities with that of the band in the single-copy reconstruction lane (rec). Neither of the bands is of the 5.5-kb size expected for the intact plasmid digested with *Hin*dIII (arrow), indicating that a rearrangement has occurred to alter the size of this fragment or that one or both of the flanking *Hin*dIII sites has been lost. Line 6K has the highest copy number of fragments homologous to *bar*, estimated to be about 35 on another gel by comparison with several reconstruction lanes (data not shown). In this and other lines with high copy numbers, most of the copies are the size of the intact plasmid cut with *Hin*dIII. In addition, these lines have many other sizes of fragments, indicative of multiple rearrangements of the transforming plasmid.

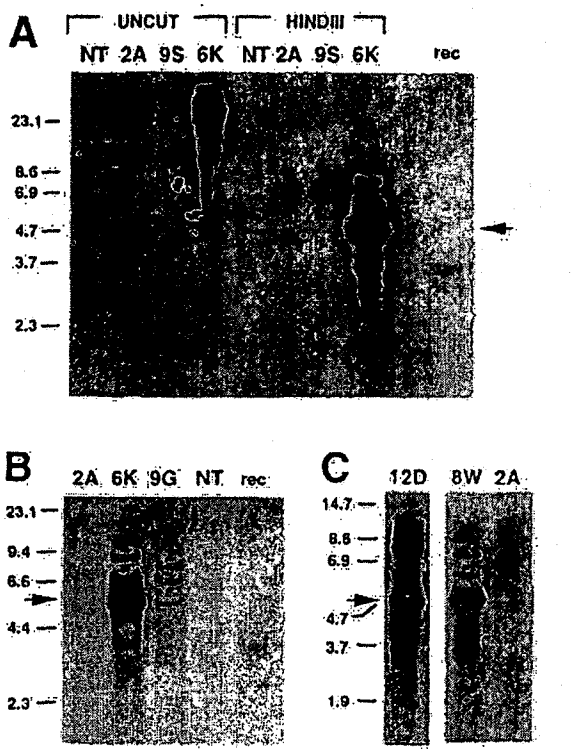

Figure 3. DNA gel blot analysis of transformants. Each lane contains 25 μg of total leaf DNA. Blots were hybridized with the coding region of the *bar* gene. Arrows mark the migration position of the 5.5-kb *Hin*dIII fragment of pAHC25 that is homologous to the *bar* probe. The migration position of markers are shown to the left of each panel with sizes in kb. A, Autoradiogram of DNA from transformed lines 2A, 9S, and 6K. The left four lanes contain undigested DNA; the middle four labeled lanes contain DNA digested with *Hin*dIII. The lanes marked NT contain DNA from nontransformed leaves. The lanes labeled "rec" contain 25 μg of DNA from nontransformed leaves and 1.6 pg of the ubiquitin:*bar* sequences of pAHC25 digested with *Pvu*I and *Xho*I (single copy reconstruction). B, Autoradiogram of *Hin*dIII-digested DNAs from transformed lines 2A, 6K, and 9G. C, Autoradiogram of *Hin*dIII-digested DNAs from transformed lines 12D, 8W, and 2A.

bar Gene Expression in Transformed Plants

One week after the regenerated plants were transferred to soil for acclimation to greenhouse conditions, the leaf tip was

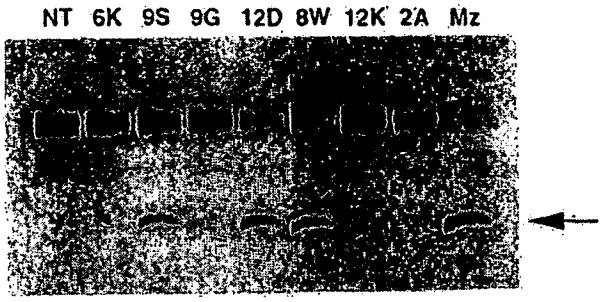

Figure 4. PAT activity in protein extracts of leaf tissue. The first lane (NT) contains an extract from a nontransformed control plant. The next seven lanes contain extracts from putative transformants selected for their ability to form roots on bialaphos-containing medium. The sample in the last lane (Mz) serves as a positive control and contains an extract from a maize callus line transformed with a plasmid containing the *bar* gene under control of the cauliflower mosaic virus 35S promoter (R.E. Williams and P.G. Lamaux, unpublished data). The position of acetylated phosphinothricin is marked by the arrow.

sampled for determination of PAT activity (Fig. 4). The level of enzyme activity, as measured by production of the acetylated form of phosphinothricin (arrow), is highest in plants 12D, 9S, and 8W, moderate in 2A, and nondetectable in 6K and 12K. The level of PAT activity is not correlated to the DNA copy number. For example, although line 9S contains high levels of enzyme activity, it has only two *bar*-homologous fragments. Line 6K contains the highest number of copies of the intact ubiquitin:*bar* *Hin*dIII fragment (Fig. 3), yet no more than background levels of PAT activity. Yet all these plants clearly exhibited bialaphos resistance at the rooting stage.

Inheritance of Bialaphos Resistance Genes

To verify inheritance of the bialaphos resistance phenotype without waiting for the seeds to mature, 38 immature embryos were excised from a control and from a 2A plant 15 DAF and plated on MS agar with or without bialaphos selection. All 19 of both the 2A and control embryos germinated into plantlets with shoots and roots on the medium lacking bialaphos (upper Fig. 2J), showing that the early development of embryos in both the transformed and nontransformed plants was normal. Nine of 19 T_1 embryos from line 2A germinated in the presence of 3 mg/L of bialaphos, whereas none of 19 nontransformed embryos germinated on the herbicide (lower Fig. 2J). Six resistant T_1 plantlets were transferred to soil and all exhibited PAT enzyme activity in leaf extracts (data not shown). To determine the segregation ratio of the resistance trait, 49 seedlings were germinated from mature T_1 seeds and sprayed 10 d later with a 2% solution of the herbicide Basta. Forty-two of the seedlings survived. Thus, the bialaphos resistance trait apparently segregated in a 3:1 ratio, indicative of single insertion site for the functional *bar* transgene(s) ($\chi^2 = 2.2$, $0.2 < P < 0.1$, with 1 degree of freedom and the Yates correction factor). T_1 plants were further tested for phosphinothricin resistance at the boot stage by application of 2% Basta (Fig. 2K). The transgenic plant was completely resistant and went on to flower and set seed, whereas the control plant was dead within 2 weeks.

To verify the co-segregation of the resistance phenotype and the transgene(s), DNA was isolated from both herbicide-resistant (PAT^+) and -sensitive (PAT^-) T_1 plants and analyzed for the presence of *bar* sequences (Fig. 5). The PAT^+ plants inherited both *bar*-homologous DNA fragments that were present in the T_0 plant, whereas sensitive plants inherited neither. Thus, in plant 2A, the plasmid DNA is integrated into the wheat nuclear genome at either a single site or at two closely linked sites.

Summary of Transformation Experiments

Table I summarizes the results of several bombardment experiments that resulted in production of callus lines or plants shown to contain detectable PAT enzyme activity and/or *bar*-homologous DNA fragments. Independent lines of stably transformed plants were produced at various times of the year, regardless of whether the embryos used for bombardments were obtained from plants grown in the short days of winter, e.g. bombardment 2, or the long days of summer, e.g. bombardment 15. Overall, the frequency of obtaining transformed fertile plants was 1 to 2 per 1000 embryos bombarded.

DISCUSSION

As summarized in Table I, we have obtained multiple independent callus lines of transgenic wheat. All but three of these have yielded one or more plants, and all but one of these plant lines were fertile. The procedure used to obtain

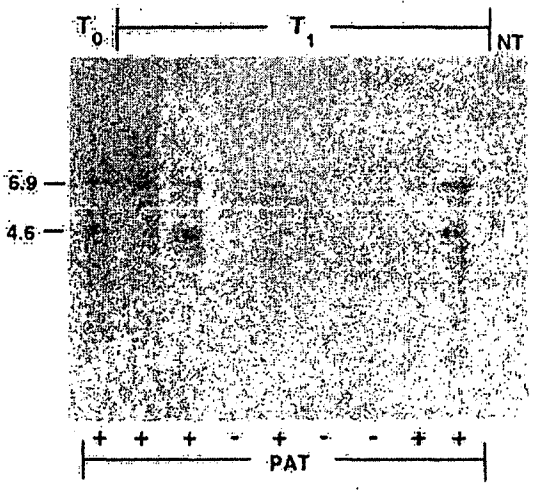

Figure 5. DNA gel blot analysis of leaf DNA from T_0 and T_1 plants of line 2A. DNAs from the parental plant (T_0), eight progeny plants (T_1), and a nontransformed control plant (NT) were digested with *Hin*dIII and hybridized to *bar*. The sizes in kb of the two *bar*-homologous fragments of parental plant 2A were deduced from other gels run with marker standards and are shown to the left. The results of the PAT enzyme assay for each 2A plant are shown below each lane.

Table I. *Summary of transformation experiments*

Bombardment[a]	Date Bombarded	No. of Embryos Bombarded	No. of Transformation Events[b]	No. of Events Producing T_0 Plants	No. of T_0 Plants
2	2-28-92	91	1	1	4
3	3-04-92	68			
4	3-24-92	342			
5	4-03-92	260			
6	4-14-92	139	1	1	1
7	4-20-92	268			
8	5-04-92	613	1	1	2
9	5-11-92	694	3	2	21
10	5-18-92	344			
11	5-26-92	417			
12	6-17-92	1043	6	5	37
14	8-17-92	664			
15	8-31-92	830	1	0	0
16	9-21-92	475			
Total		6248	13	10	65

[a] Bombardment 1 was used for transient expression experiment and bombardment 13 was lost to contamination. [b] As determined by resistance of tissue to bialaphos at the callus and rooting stage and the presence of either PAT enzyme activity or DNA homologous to *bar* coding sequence.

transformants has several advantages over that previously published for wheat. It is reproducible in our hands, yielding 1 to 2 transgenic plant lines per 1000 embryos bombarded. It is relatively rapid. The target tissue takes only 5 d to establish in culture, in contrast to the time-consuming and labor-intensive process needed to establish regeneration-competent cell suspension or callus cultures. On the average, about 5.5 months (168 d) elapsed between excision of embryos for bombardment and anthesis of the transformed T_0 plants. There are two main sources of variation in the time it takes to produce transgenic progeny. One is the season of the year, which determines the length of time for the various growth stages in the greenhouse. The second is the regeneration process. Green sectors arise at variable times after the resistant callus lines are established. The shortest time we have observed thus far between bombardment and the identification of a transformed plantlet in the rooting assay is 75 d.

Our results show that, among the various phenotypic criteria used to assess transformation, the ability of the plantlets to root in the presence of bialaphos was the most reliable indicator of stable integration of the *bar* transgene. Each plant analyzed thus far that exhibited resistance at the rooting stage contains DNA fragments homologous to the *bar* coding region. In contrast, results of the PAT assay were not strictly indicative of transformation and must be interpreted with caution. Whereas a positive PAT assay demonstrated the presence of stably integrated *bar* genes, a lack of PAT activity was not necessarily indicative of the absence of such genes. Protein extracts from nontransformed wheat leaves and calli contain a background activity that acetylates phosphinothricin at a low level (not visible in this reproduction of Fig. 4). Our results indicate that the PAT enzyme assay cannot detect activity below this threshold even though enough enzyme is present in plantlets and callus to confer resistance to phosphinothricin.

Our transgenic plant lines exhibited a range of fertility. All the lines except 6K yielded some seed without outcrossing or embryo rescue, but only 2A had completely normal levels of seed set. It will be interesting to see whether the partial fertility trait is the result of the selection process and thus limited to the T_0 generation or is exhibited by the T_1 progeny of these plants. The latter result would be indicative of an effect of the presence of the transgene on fertility. In the T_0 plants, reduced seed set is not correlated with high expression levels of the transgene, since the sterile line 6K contains no more than background levels of PAT enzyme activity.

This is the third report of the use of the maize *Ubi1* promoter in cereal transformation vectors. Plasmids containing ubiquitin:GUS and ubiquitin:*bar* were used by Cornejo et al. (1992) and Toki et al. (1992), respectively, to identify transformed callus lines in rice. Here we show that this promoter/gene combination can be used to identify wheat transformants. The ubiquitin:*bar* transgenes must be expressed in a wide variety of cell types in wheat, since phosphinothricin resistance is exhibited at several different stages of development: callus tissue, shoot and root regeneration, seedlings, and mature plants. The presence of the ubiquitin:GUS portion of pAHC25 in the genomes of these wheat plants has not yet been assessed. Transgenic plants with functional copies of ubiquitin:GUS could be used to examine in detail the tissue specificity and developmental timing of expression of the maize *Ubi1* promoter in wheat.

Several features of the transformation procedure described in this report will facilitate its adoption by other laboratories. The procedure uses standardized conditions for DNA delivery and the commercially available Biolistics device. Thus, no special apparatus need be constructed to reproduce these results. Bobwhite is a publicly available hard white spring wheat. Excision and culturing of immature embryos requires practice, but no particular tissue culture expertise. Furthermore, the use of this tissue as a target for transformation is

probably applicable to other cultivars that can be reliably regenerated from immature embryo-derived callus. Improved culture methods for some of these cultivars, described recently by Perl et al. (1992), should broaden the applicability of these techniques to even more genotypes. We are currently applying our procedures to transformation of a hard red winter wheat cultivar.

The efficiency and reliability of these procedures makes feasible, for the first time, studies of promoter and protein function in transgenic wheat. Transformation methodology will also allow a biotechnological approach to modification of important traits of wheat, including pathogen resistance and bread-making quality.

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EXHIBIT 4

US006995016B2

(12) **United States Patent**
Eudes et al.

(10) **Patent No.: US 6,995,016 B2**
(45) **Date of Patent: Feb. 7, 2006**

(54) **PROCESS FOR INDUCING DIRECT SOMATIC EMBRYOGENESIS IN IMMATURE SCUTELLA CELLS OF POOIDEAE, AND RAPIDLY REGENERATING FERTILE PLANTS**

(75) Inventors: **Francois Andre Germain Eudes**, Lethbridge (CA); **Andre J. Laroche**, Lethbridge (CA); **Surya Narayan Acharya**, Lethbridge (CA)

(73) Assignee: **Her Majesty the Queen in right of Canada, as represented by the Minister of Agriculture and Agri-Food**, Lethbridge (CA)

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(21) Appl. No.: **09/929,831**

(22) Filed: **Aug. 14, 2001**

(65) **Prior Publication Data**

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Related U.S. Application Data

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(51) **Int. Cl.**
C12N 15/82 (2006.01)

(52) **U.S. Cl.** **435/430.1**; 435/430.1; 800/278

(58) **Field of Classification Search** 435/424, 435/430.1

See application file for complete search history.

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(57) **ABSTRACT**

A process is provided for inducing direct somatic embryogenesis in Pooideae and rapidly regenerating fertile plants by first culturing isolated immature scutella cells in culture medium comprising auxin, cytokinin and polyamine in amounts effective to cause direct formation of primary embryos without an intervening callus stage, at least until at least one primary embryo reaches the globular developmental stage, the auxin being present in greater proportion than cytokinin. A second step includes either a) culturing the primary embryos under conditions to regenerate plantlets, and culturing the primary embryos in regeneration medium; or b) culturing the primary embryos at the globular developmental stage and no longer than the coleoptilar stage in culture medium comprising auxin, cytokinin, and polyamine in amounts effective to cause induction of secondary embryo formation, at least until secondary embryogenesis is detected, the cytokinin being present in greater proportion than auxin, and culturing the secondary embryos under conditions to regenerate plantlets.

56 Claims, 3 Drawing Sheets

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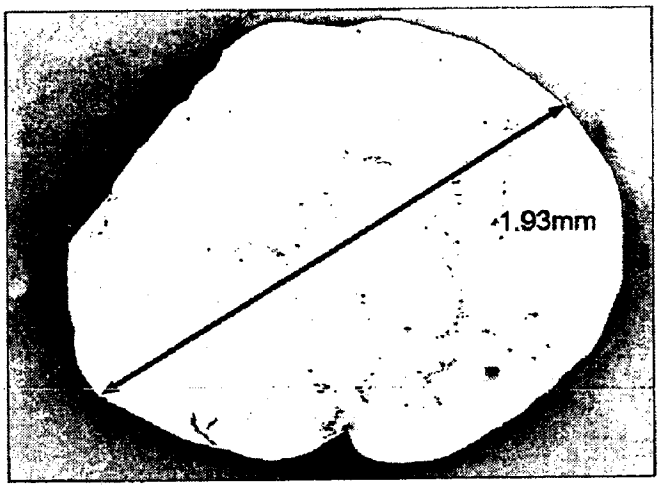


FIG. 1

FIG. 2

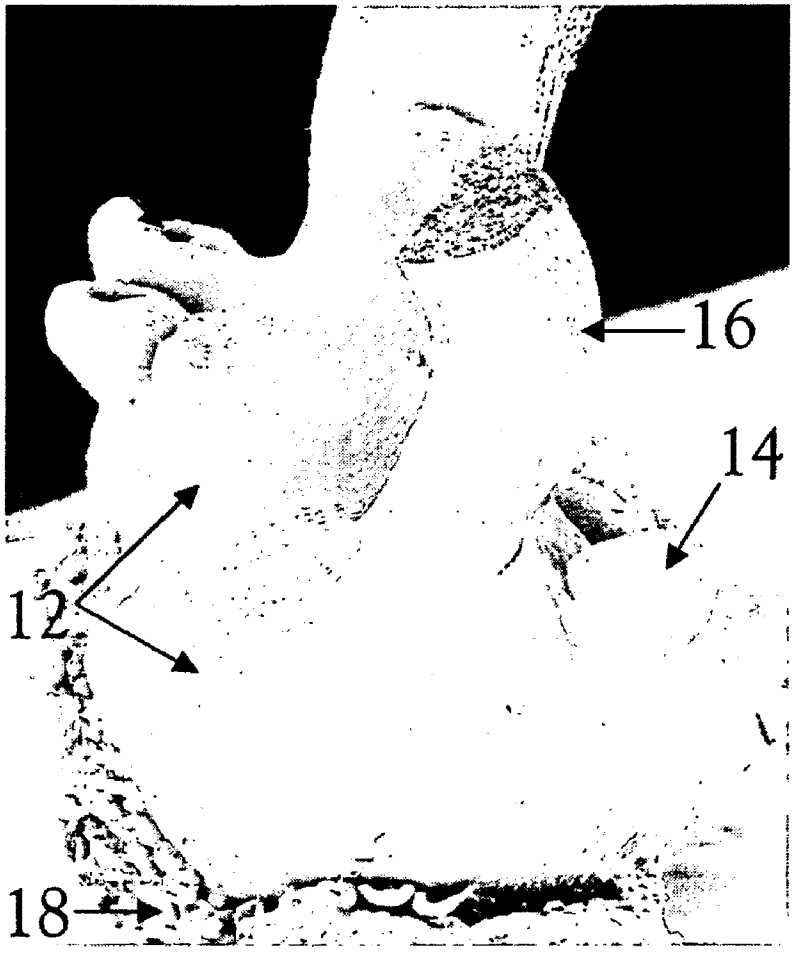
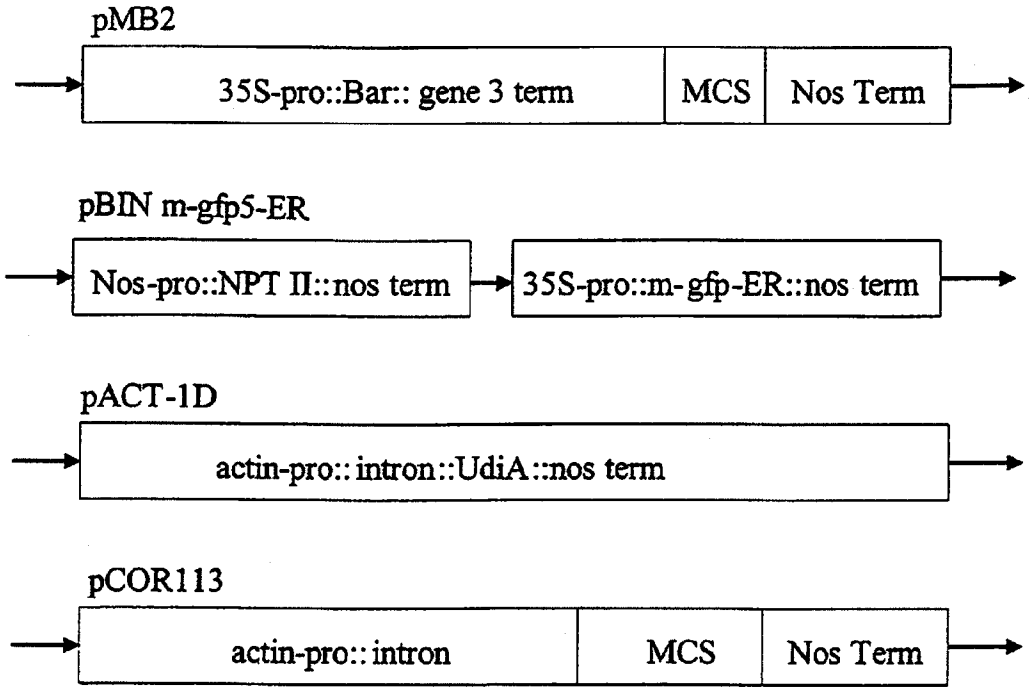


FIG. 3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pACT-1D
actin-pro::intron::UdiA::nos term
pCOR113
actin-pro::intron
MCS
Nos Term

FIG. 4

PROCESS FOR INDUCING DIRECT SOMATIC EMBRYOGENESIS IN IMMATURE SCUTELLA CELLS OF POOIDEAE, AND RAPIDLY REGENERATING FERTILE PLANTS

CROSS-REFERENCE TO RELATED APPLICATION

This application is a Continuation in Part of U.S. patent application Ser. No. 09/641,243, filed Aug. 17, 2000, now abandoned, which is incorporated by reference herein to the extent that there is no inconsistency with the present disclosure.

FIELD OF THE INVENTION

The invention pertains to plant tissue culture techniques. In particular, the invention relates to processes for inducing direct somatic embryogenesis and secondary embryogenesis in monocotyledonous plant cells, and rapidly regenerating fertile plants.

BACKGROUND OF THE INVENTION

The introduction of new traits by gene transfer has become routine with respect to many dicotyledonous plant species. In contrast, monocotyledonous plant species, particularly cereals, have proven to be recalcitrant to genetic engineering because they do not belong to the natural host range of *Agrobacterium*. Therefore, unlike dicots, monocots are generally not susceptible to gene transfer by *Agrobacterium*-mediated transformation. However, the application of such methods as electroporation, PEG-mediated transformation of protoplasts, and particle bombardment, have shown promise for the stable transformation of cereals. Moreover, successful transformation of monocots using hypervirulent *Agrobacterium* strains has been recently reported.

However, the step of the introduction of foreign DNA into plant cells is only part of the equation for obtaining plants that possess new traits. It is also necessary that the transformed cells be successfully regenerated to form viable plants. Despite advances in methods for transforming monocots with foreign DNA, regeneration of fertile plants from transformed somatic cells or tissues remains a challenge, particularly in barley and wheat. One problem is that, in monocotyledonous plants, only a few somatic tissues are totipotent, i.e. capable of being regenerated to form green, fertile plants. Plants can be regenerated from totipotent cells in tissue culture by either embryogenesis or organogenesis.

i) Embryogenesis

TABLE 1

Comparison of zygotic and somatic embryogenesis (based on Dodeman et al., 1997)

Steps	Zygotic embryogenesis	Somatic embryogenesis
Origin	Zygote In the ovule	Somatic cell Isolated or not Haploid cells (e.g. microspore)
Initiation	Fecundation (except apomixis) Every zygote Constitutional polarity	Hormonal induction Low cell frequency Dedifferentiation Polarity?

TABLE 1-continued

Comparison of zygotic and somatic embryogenesis (based on Dodeman et al., 1997)

Steps	Zygotic embryogenesis	Somatic embryogenesis
	Asymmetric division (under genetic control)	Asymmetric division?
Construction of an embryo	Embryo/suspensor Embryo axis in place (under genetic control)	Similar conditions, but with variations: Absence of the suspensor Reorganization in proembryo clusters Adventitious embryogenesis
Meristem formation	Tightly genetically controlled root meristem shoot meristem	Interaction between genetic and hormonal controls Numerous abnormalities
Maturation	Storage protein Dehydration Dormancy (genetic control, ABA) Plant maternal tissue-embryo interaction	Absence of maturation and endosperm External induction factors (amino acids, sugars, ABA, dehydration)

Embryogenesis is the process of embryo initiation and development, and may be classified as either zygotic or somatic, according to the type of cell from which embryogenesis arises. Features of zygotic and somatic embryogenesis are summarized in Table 1 (based on Dodeman et al., 1997).

Zygotic embryogenesis relates to embryogenesis arising from the zygote or fertilized egg which originates in the ovule, and is intrinsically embryogenic. Proliferation of the zygote leads to the formation of a zygotic embryo within which organs and tissues begin development. Within the embryo, distinctive regions, designated as the shoot and root apical meristems, promote the development of shoot and root systems respectively. Activity of the meristems contributes to the continuing expansion and formation of the plant. During the maturation stage, storage protein accumulates and dehydration enhances germination. In zygotic embryogenesis, the cells are restricted to specific fates since the developmental stages (i.e. globular, heart, torpedo, cotyledonary) are strictly genetically controlled.

In comparison, somatic embryogenesis relates to embryogenesis arising from somatic cells (i.e. vegetative or non-gametic cells), namely from isolated somatic explants or microspores. Since somatic cells are not naturally embryogenic, such cells must be induced to become embryogenic. Conversion from somatic to embryogenic cells may be achieved by external stimuli such as auxin, cytokinin, pH shifts, growth regulators, and heavy metal ions (Yeung, 1995; Dodeman et al., 1997).

Successful formation of somatic embryos is largely dependent upon the explant tissue of choice (Merkle et al., 1995). Very young zygotic embryos form somatic embryos in response to cytokinin, whereas more mature zygotic embryos no longer respond to cytokinins alone and require auxin to form somatic embryos. Meristematic cells from grasses and other monocots behave similarly. In more differentiated tissue, auxin and cytokinin induce formation of calli from which somatic embryos may be produced.

Regardless of the explant source, the obtained somatic embryo may then follow a developmental pattern similar to that of a zygotic embryo. However, unlike zygotic embryogenesis in which the developmental fate of cells is programmed, somatic embryogenesis differs in that variations and abnormalities may arise during the stages of embryo construction and meristem formation. Variations observed in

vitro include the absence of the suspensor; reorganization of cells in proembryo clusters; occurrence of adventitious embryogenesis; and other abnormalities due to interaction between genetic and external hormonal controls. In zygotic embryos, activity of the meristems is crucial towards the development of the plant, whereas in somatic embryos, little is known about meristem differentiation. In comparison to zygotic embryos, maturation and storage reserves are absent in somatic embryos. Notably, somatic embryos tend to germinate precociously, with abnormalities observed such as failure or uncoordination of shoot or root formation, multiple cotyledons, or precocious or abnormal shoot formation (Wetherell, 1979). Such abnormalities are not considered problematic since in general, plantlets with normal shoots and roots eventually form (Wetherell, 1979). The fate of the cells in somatic embryos is thus not as fixed as that of cells of zygotic embryos, which follow a determined and highly regulated pathway. However, somatic embryos are beneficial for their abilities to form a complete plant despite the natural mutations that may occur in vitro, and to germinate precociously, contributing to rapid regeneration of plants.

ii) Organogenesis

As an alternative to embryogenesis, plants may also be regenerated from totipotent cells in tissue culture by organogenesis, whereby new organs such as shoots and roots, rather than whole embryos as in embryogenesis, form directly from cultured cells. The process can occur directly on the explant or indirectly via calli formation. A significant feature of organogenesis is the development of a meristem or shoot/root primodium. Only one meristem is formed in organogenesis, while two meristems (one for a shoot and the other for a root) are produced in embryogenesis. However, it is not uncommon to encounter a zygotic embryo with only one meristem (a shoot primordium) upon dissection of an immature barley embryo.

At the physiological, biochemical and structural levels, embryogenesis and organogenesis have certain common features; thus, in morphogenetic in vitro studies, it may be difficult to confirm whether somatic embryogenesis or true organogenesis has occurred (Thorpe, 1993).

Due to variations which naturally occur with somatic embryos and factors such as the explant source, media, or tissue culture technique, cells may have different developmental fates, such that some cells produce embryos (i.e. embryogenesis), while others form shoot or root primordia (i.e. organogenesis).

Although both organogenesis and embryogenesis lead to regeneration of fertile plants, embryogenesis has certain advantages. First, somatic embryos, like zygotic embryos, naturally proceed through the developmental process to form a complete plant, with little intervention. In contrast, during organogenesis, separate shoot growth and rooting steps are usually required in order to obtain complete plantlets. Second, when cultured under appropriate conditions, rather than proceeding to the next developmental stage, a somatic embryo may instead give rise to new somatic embryos. This process has been described as secondary, recurrent, or repetitive embryogenesis. Because somatic embryos can be perpetuated via repetitive embryogenesis, they are attractive candidates for the mass production of clonal plantlets.

In monocot tissue culture, the most commonly used regenerable embryogenic tissues are embryogenic suspension cells and embryogenic callus cultures. Suspension cultures are substantially homogeneous suspensions of microcalli in liquid medium. Callus cultures are grown on solid media, develop larger contiguous masses of calli, and are more heterogeneous with respect to the embryogenic quality of the calli. Both suspension cells and calli have limitations as target tissues for transformation with foreign DNA. The preparation of suspension cells involves a lengthy in vitro culture period, and the cells exhibit a significant reduction of morphogenetic competence over time. Additionally, plants regenerated from suspension cells manifest substantial undesirable somaclonal variation, such as infertility or albinism.

Because the time needed for establishment of culture and plant regeneration is shorter than with suspension cells, embryogenic callus has been considered as possibly a preferable target tissue, but problems remain. Somaclonal variation persists, and most cells lose their ability to regenerate when they reach the callus stage (Jähne et al., 1995). Despite efforts to improve callus culture, only a few monocot genotypes can be successfully regenerated from calli. In North America, the small number of genotypes which have been used successfully to regenerate fertile plants from calli include the barley genotype Golden Promise, the winter barley genotype Igri, and the wheat genotypes Fielder, Bobwhite, and Chinese Spring. Moreover, cereal calli remain embryogenic only briefly, further compounding the difficulties experienced in tissue culture.

In view of the limitations of suspension cells and callus cultures, efforts have been directed towards using primary explants such as immature embryos and inflorescences as target tissues for obtaining stably-transformed plants. However, the tissue culture techniques which have been applied to primary explants result in indirect somatic embryogenesis, wherein the intermediate cell generations between the original explant and the formation of somatic embryos are manifested as calli. Hence, indirect somatic embryogenesis from primary explants does not entirely resolve the problems associated with regeneration of plants from callus cultures.

Typically, indirect somatic embryogenesis in tissue culture involves two distinct steps, induction and regeneration. During induction, the tissue of interest is cultured on an induction medium which encourages un-differentiation of cells, and induction of fast-growing embryogenic calli. The callus stage is characterized by rapid, anarchic cell division. Tissues are cultured on the induction medium for a fixed, predetermined period, which is of sufficient duration for the production of fast growing embryogenic calli. This period typically ranges from one to four weeks (Nehra et al., 1994; Becker et al., 1994). If necessary, the tissue may be subcultured on the same medium for an additional period of time (Cho et al, 1998).

The hormone content of the media is of greatest significance. The three major classes of plant growth regulators used in tissue culture are auxins, cytokinins, and polyamines. Auxins are involved in many aspects of cell biology and tissue development. The most common are the naturally-occurring auxins indole-3-acetic acid (IAA), indole butyric acid (IBA), phenylacetic acid (PAA), and the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D), dicamba (2-methoxy-3,6-dichlorobenzoic acid) and picloram (4-amino-3,5,6-trichloropiconilic acid). Like auxins, cytokinins are involved in many aspects of cell biology and tissue development, especially cell division. The naturally-occurring cytokinins benzyl amino purine (BAP), benzyladenine (BA) and zeatin, and the synthetic cytokinin kinetin are the most commonly used in tissue culture. Polyamines, which include spermine, spermidine and putrescine, are less well known than other plant growth regulators. Although

their precise physiological role still remains to be determined, polyamines appear to influence cell division and embryogenesis in carrot cell culture. They also bind to nucleic acids, phospholipids and proteins to further stabilize these molecules.

A typical callus induction medium for barley and wheat is Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), which itself is hormone free, supplemented with 30 g/L maltose, 1.0 mg/L thiamine-HCl, 0.25 g/L myo-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L proline, and 11.3 μM of dicamba (Wan and Lemaux, 1994). Other variants of basal MS medium are also known. For instance, Cho et al. (1998) describe an induction medium containing 4.5–11.3 μM 2,4-D, and 2.2 μM BAP. Nehra et al. (1994) and Bregitzer et al. (1998) teach induction media in which 9-13.6 μM 2,4-D is the only hormone component. Ritala et al. (1994) provide an induction medium in which the only plant hormone is 1.8 μM BAP. Barro et al. (1998) report that, depending on the conditions and tissues, the presence of 16.6 μM picloram can result in higher transformation efficiency than the presence of 2,4-D alone. Similarly U.S. Pat. No. 5,631,152 to Fry et al. teaches an induction medium containing 9.1 μM picloram and 2.2 μM 2,4-D.

Another basal medium commonly used for induction of callus culture is hormone free L3 medium supplemented with 30 g/L maltose and 9 μM of 2,4-D (Barcelo et al., 1993). The medium commonly used for induction of barley microspores culture is quite similar, and contains FHG basal medium supplemented with 63 g/L maltose, 730 mg/L glutamine, 100 mg/L myo-inositol, 0.4 mg/L thiamine-HCl, 4.4 μM BAP and 73.4 μM PAA (Yao et al., 1997).

In the second step, the calli are cultured on a regeneration medium such as MS, FGH, or L3. The regeneration medium is usually hormone free, though it may be supplemented with a very small amount of cytokinin and auxin, in the order of less than 4.5 μM. Termination of the auxin-mediated hormonal control allows embryogenesis to commence. As they mature, developing embryos produce shoots and regenerated plantlets. If necessary, the mass of cells with green shoots is excised and placed on a rooting medium. Rooting media typically do not contain plant hormones, although some may contain up to about 2 μM of auxin. The plantlets are then transferred to soil.

Although the two-step induction and regeneration approach to somatic embryogenesis has been applied to monocots, it has a number of significant disadvantages. First, since the induction step involves proliferation of calli, somaclonal variation remains a concern. Second, induction and regeneration are slow. Since the culture steps proceed according to a pre-determined time line, there is no opportunity to proceed more rapidly should the tissue reach the next developmental stage more quickly than anticipated. Generally, induction of calli and regeneration of green, fertile plants by indirect somatic embryogenesis takes at least three months.

In contrast to indirect somatic embryogenesis, a tissue culture process for direct somatic embryogenesis in monocotyledonous plants would advantageously avoid the callus step, thereby minimizing somaclonal variation. Moreover, such a process would also desirably eliminate the constraints of a pre-determined tissue culture schedule, thereby enabling plant regeneration to proceed as quickly as is biologically feasible. Direct somatic embryogenesis has been reported in dicots such as clover, carrot, and tobacco. For instance, Maheswaran and Williams (1984, 1985) disclose direct somatic embryogenesis of immature embryos of *Trifolium repens* (white clover), *Trifolium pratense* (red clover), and *Medicago sativa* (alfalfa) cultured on a basal nutrient medium (EC6) supplemented with 0.22 μM of the cytokinin BAP.

Despite reports of direct somatic embryogenesis in dicots, to the applicants' knowledge, direct somatic embryogenesis has not been accomplished in monocots. The patent literature discloses a number of methods for somatic embryogenesis in monocotyledonous plant tissues, but these involve a step of inducing calli, and therefore constitute indirect, rather than direct somatic embryogenesis. For instance, U.S. Pat. No. 5,631,152 to Fry et al. teaches indirect somatic embryogenesis in *Triticum aestivum*. U.S. Pat. Nos. 5,641,664 and 5,712,135 to D'Halluin et al. and U.S. Pat. No. 5,792,936 to Dudits et al., teach regeneration of corn plants from calli cells. U.S. Pat. No. 5,589,617 to Nehra et al. teaches a method for regenerating plants from wheat or barley embryos, which involves the induction of a callus stage. U.S. Pat. No. 5,610,042 to Chang et al. teaches a method for producing stably transformed fertile wheat plants involving a step of inducing formation of calli from immature wheat embryos. U.S. Pat. No. 5,874,265 to Adams et al. teaches production of stable, genetically transformed cereal plants, particularly wheat, barley, or oats, in which regeneration of plants from the transformed cells involves induction of calli. U.S. Pat. No. 4,666,844 to Cheng provides a process for regenerating cereal plants such as barley, corn, wheat, rice, and sorghum, in which tissues are first cultured under conditions sufficient to ensure calli formation. U.S. Pat. No. 5,981,842 to Wu et al. teaches the regeneration of transgenic rice (*Oryza sativa*), from calli induced from immature embryos. U.S. Pat. No. 5,409,828 to Frenkel et al. describes somatic embryogenesis in *Asparagus officinalis*, which is a monocot. But again, the first step is the induction of calli. Canadian Patent No. 1,292,959 to Stuart et al. describes somatic embryogenesis of corn and rice, again involving a callus step. International Publication No. WO 99/04618 to Rikiishi et al. discloses a method for producing transformed barley cells, which includes the step of culturing the barley cells in a calli induction medium.

Jähne et al. (1994) report some success in obtaining direct embryogenesis in barley microspores. However, microspores are germ cells, rather than somatic cells. Like other isolated germ cells, microspores are delicate relative to somatic cells, and they are very susceptible to damage by particle bombardment. Jähne et al's process is therefore of limited utility for introducing foreign genes into monocots, given that particle bombardment is the preferred transformation technique in monocots. Similarly, U.S. Pat. Nos. 5,322,789 and 5,445,961 to Genovesi et al. describe culturing corn microspores to obtain embryoids or calli.

Hence, there is a need for a tissue culture process suitable for effecting direct somatic embryogenesis in monocotyledonous plant cells or tissues and rapid regeneration of fertile plants. Ideally, in order to expedite the recovery of fertile plants, such a process would not impose predetermined time limits on the various tissue culture steps. Such a process would advantageously provide for recurrent or secondary embryogenesis from the developing embryos. In addition, such a process would also promote organogenesis in developing embryos. A direct somatic embryogenesis method for monocots would also desirably provide for the ready introduction of foreign genes into the plant.

SUMMARY OF THE INVENTION

The invention provides rapid and efficient processes for inducing direct somatic embryogenesis and secondary

embryogenesis in monocotyledonous plants, particularly recalcitrant plant species such as wheat and barley, and thereafter regenerating fertile plants. In contrast to prior art tissue culture methods involving indirect somatic embryogenesis, direct somatic embryogenesis avoids a callus step, and its attendant problems, such as increased somaclonal variation. Tissue culture steps of the invention progress on the basis of the developmental stage of the cultured cells, rather than in accordance with a pre-determined time line, thereby providing green, fertile plants more rapidly than do previous tissue culture methods. In the first step, embryogenic monocotyledonous plant cells are cultured under conditions conducive to direct formation of primary embryos without an intervening callus stage. The cells are not cultured for a pre-determined period of time, but rather until a desired developmental stage is detected by observation of the cells. Preferably, the cells are cultured in the first step for a period of time sufficient for at least one primary embryo to reach the globular developmental stage. More preferably, the cells are cultured until primary embryogenesis is substantially complete, and most of the primary embryos have reached the globular developmental stage. In a second step, one or more of the globular-stage primary embryos from the first step are cultured under conditions conducive to induction of secondary embryo formation, at least until secondary embryogenesis is detected. Advantageously, the primary embryo(s) are cultured in the second step until secondary embryogenesis is well established. In a third step, the one or more secondary embryos from the second step are cultured under conditions conducive to regeneration of plantlets from the secondary embryos. Thus, in one aspect, the invention provides a process for inducing direct somatic embryogenesis and secondary embryogenesis in monocotyledonous plant cells, and rapidly regenerating fertile monocotyledonous plants, comprising the steps of:

(a) culturing embryogenic monocotyledonous plant cells under conditions conducive to direct formation of primary embryos without an intervening callus stage, at least until at least one primary embryo reaches the globular developmental stage and no longer than the coleoptilar stage of the primary embryo;

(b) culturing one or more of the primary embryos from step (a) under conditions conducive to induction of secondary embryo formation, at least until secondary embryogenesis is detected; and

(c) culturing one or more of the secondary embryos from step (b) cells under conditions conducive to regeneration of plantlets from the secondary embryo.

The step of secondary embryogenesis circumvents the problem of chimeric embryos by allowing recovery of completely transformed secondary embryos from transformed sectors within a primary somatic embryo. Even if chimeric embryos are still recovered from the first cycle of secondary embryogenesis, continued cycling in the presence of a selective agent eventually results in embryos consisting entirely of transformed cells. There may be instances, however, wherein the recovery of chimeric embryos is acceptable. In such cases, the step of secondary embryogenesis can be eliminated. Thus, in another aspect, the invention provides a process for inducing direct somatic embryogenesis in monocotyledonous plant cells and rapidly regenerating fertile monocotyledonous plants, comprising the steps of:

(a) culturing embryogenic monocotyledonous plant cells under conditions conducive to direct formation of primary embryos without an intervening callus stage, at least until at least one primary embryo reaches the globular developmental stage; and

(b) culturing one or more of the globular-stage primary embryos from step (a) under conditions conducive to regeneration of plantlets from the primary embryo.

Due to variations which naturally occur with somatic embryos and factors such as the species of plant and explant source, cells may have different developmental fates, such that some cells produce embryos (i.e. embryogenesis), while others form shoot or root primordia (i.e. organogenesis), as discovered by the present inventors. In particular species, organogenesis may arise following culturing of globular-stage primary embryos obtained from direct somatic embryogenesis. Organogenesis, as detected by at least the formation of adventitious shoots, occurs in species such as sorghum and corn as demonstrated in Example 4. The adventitious shoots are then cultured to regenerate plantlets. Thus, in another aspect, the invention provides a process for inducing direct somatic embryogenesis and organogenesis in monocotyledonous plant cells of particular plant species and rapidly regenerating fertile monocotyledonous plants, comprising the steps of:

(a) culturing embryogenic monocotyledonous plant cells under conditions conducive to direct formation of primary embryos without an intervening callus stage, at least until one primary embryo reaches the globular developmental stage and no longer than the coleoptilar stage of the primary embryo;

(b) culturing one or more of the primary embryos from step (a) under conditions conducive to induction of organogenesis, at least until adventitious shoots are detected; and

(c) culturing one or more of the adventitious shoots from step (b) under conditions conducive to regeneration of plantlets.

The foregoing aspects of the invention are directed principally to inducing direct somatic embryogenesis in primary explants such as, without limitation, immature embryos, meristems, and inflorescenses. However, aspects of the invention may also be used for rapidly inducing embryogenesis in embryogenic monocotyledonous callus cells, suspension cells, or microspore-derived embryos. In such instances, the step of direct somatic embryogenesis is omitted, and the callus cells, suspension cells, or microspore-derived embryos are cultured first in the presence of such amounts of plant hormones that would otherwise be used to induce secondary embryogenesis. Hence, in another aspect, the invention provides a process for inducing somatic embryogenesis in monocotyledonous callus cells, suspension cells, or microspore-derived embryos, and rapidly regenerating fertile monocotyledonous plants, comprising the steps of:

(a) culturing embryogenic monocotyledonous callus cells, suspension cells or microspore-derived embryos in or on a culture medium comprising auxin, cytokinin, and polyamine in amounts effective to cause induction of embryo formation, the cytokinin being present in greater proportion than the auxin, at least until at least one embryo reaches the globular developmental stage; and

(b) cultivating one or more of the globular-stage embryos from step (a) under conditions conducive to regeneration of plantlets.

The invention also extend to the use of preferred culture media with specific plant hormones for the various stages of the embryogenesis processes set out above.

Also provided are fertile monocotyledonous plants produced according to the foregoing processes, and methods of

transforming such plants to introduce foreign DNA so that the foreign DNA becomes stably integrated into the genome of the embryogenic cells.

As used herein and in the claims, the terms and phrases set out below have the meanings which follow.

"Embryogenesis" means the process of embryo initiation and development.

"Embryogenic," in the context of cells or tissues, means that the cells or tissues can be induced to form viable plant embryos, under appropriate culture conditions.

"Explant" means tissue taken from its original site and transferred to an artificial medium for growth or maintenance.

"Induction" means initiation of a structure, organ or process in vitro.

"Regeneration" means a morphogenetic response to a stimulus that results in the production or organs, embryos, or whole plants.

"Germination" means the growth of leaves and roots from the germ or embryo.

"Plantlet" means a small regenerated plant with green shoots and roots.

"Callus" or "calli" means a mass of unorganized tissues made up of undifferentiated cells that normally divide very rapidly.

"Suspension cells" or "suspension culture" refers to a substantially homogeneous suspension of microcalli in liquid medium.

"Microspore-derived embryo" refers to an embryo which arises from an induced microspore in anther culture or isolated microspore culture.

"Auxin" is meant to include naturally-occurring auxins such as, without limitation, indole-3-acetic acid (IAA), IAA conjugated with an amino acid, indole 3-butyric acid (IBA), indole 3-butyric acid-potassium salt (KIBA), phenylacetic acid (PAA), and synthetic auxins such as, without limitation, 2,4-dichlorophenoxyacetic acid (2,4-D), dicamba (2-methoxy-3,6-dichlorobenzoic acid), picloram (4-amino-3,5,6-trichloropiconilic acid), 4-chlorophenoxyacetic acid (CPA), α-napthaleneacetic acid (NAA), and β-napthoxyacetic acid (NOA), or combinations thereof.

"Cytokinin" is meant to include naturally occurring cytokinins such as, without limitation, benzyl amino purine (BAP), 6-benzylaminopurine (BA), 6-benylaminopurine riboside (BAR), 6-(γ,γ-dimethylallylamino) purine (2iP), 6-(γ,γ-dimethylallylamino) purine riboside (2iP-R), N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU) zeatin, zeatin riboside (ZR), dihydrozeatin, and thidiazuron, and synthetic cytokinins such as, without limitation kinetin and kinetin riboside (KR), or combinations thereof.

"Polyamine" is meant to include, without limitation, the plant growth regulators spermine, spermidine, putrescine, and cadaverine.

"Somatic embryogenesis" means the formation of embryos from vegetative (or non-gametic tissues) with 2n chromosomes, rather than by sexual reproduction.

"Direct somatic embryogenesis" means a form of embryogenesis wherein embryos develop from vegetative cells without an intervening callus step or stage.

"Indirect somatic embryogenesis" means a form of embryogenesis wherein embryos develop from callus tissues induced in vitro.

"About," when used to describe a concentration of auxin, cytokinin, or polyamine, means ±10%.

"Primary embryogenesis" is meant to refer to a first stage or cycle of somatic embryogenesis, in which the embryos which are formed are referred to as "primary embryos."

"Secondary embryogenesis" is meant to refer to a second stage or cycle of somatic embryogenesis to give rise to new somatic embryos, which are termed "secondary embryos."

"Organogenesis" is meant to refer to induction of new organs, such as new adventitious shoots or roots.

"Adventitious" means developing from unusual points of origin, such as shoots or root tissues from calli, or embryos from sources other than zygotes.

A medium that is "essentially free" of auxin or cytokinin means a medium that, if auxin and/or cytokinin is present at all, it is present at a sufficiently low level that it does not effect hormonal control over the cultured cells.

"Foreign DNA" and "foreign gene," when used in the context of transforming target plant cells, is meant to encompass not only DNA which originates from sources other than the target plant, but also encompasses DNA which originates from the target plant but that has been introduced or manipulated "by the hand of man" such that it exists in an arrangement or juxtaposition other than it exists in nature (e.g. such as DNA inserted into a vector).

"Molecular farming" means the use of transgenic plants to produce large quantities of a desired gene product.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1** and **2** are scanning electron microscopy images of barley scutella at various stages of direct somatic embryogenesis and secondary embryogenesis, in which:

FIG. **1** shows an immature scutellum at the time of culture initiation, prior to the commencement of embryogenesis.

FIG. **2** shows the immature scutellum after five days of culture, at which time direct somatic embryogenesis has commenced. Globular primary embryos **12** and a small amount of calli **10** are observed on different tissue parts of the scutellum.

FIG. **3** is a scanning electron microscopy image depicting stages of direct somatic embryogenesis and secondary embryogenesis in wheat, showing germinating primary embryos **12** with secondary embryos **14** arising at the equatorial plane, and primary leaves **16** and a small amount of tissue remaining from the scutellum **18**.

FIG. **4** illustrates various gene constructs used in the Examples herein.

DETAILED DESCRIPTION OF THE INVENTION

i) Isolation of Primar Explants

The processes of the invention are useful for inducing direct somatic embryogenesis in a wide range of monocotyledonous plants. Monocotyledonous plants (i.e. monocots) are distinguished from dicotyledonous plants (i.e. dicots) principally on the basis that the seed of a monocot contains a single cotyledon, whereas that of a dicot contains two cotyledons. The invention is of particular benefit when applied to monocotyledonous plants within the Poaceae family. These plants have proven to be recalcitrant to other tissue culture techniques. Preferred Poaceae include common wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), *Triticum monococum, Triticum urartu*, barley (*Hordeum vulgare*), rye (*Secale cereale*), oat (*Avena sativa*), triticale, corn (*Zea mays*), rice (*Oryza sativa*), sorghum (*Sorghum vulgare*), millet (*Pennesitum glaucum* and *Pennesitum purpureum*), and sugarcane (*Saccharum officinale*), many of which have responded poorly in the past to tissue culture. The invention can also be applied to other monocots,

including, without limitation: members of the Liliaceae family, particularly members of the genus *Allium*, such as garlic, leek, onion, and chive; and other members of the Poaceae family, particularly members of the genera *Dactylis* (e.g. orchard grass), *Bromus* (e.g. brome), and *Lolium* (e.g. perennial rye grass), or festuca. The processes of the invention are genotype independent, and can be applied, in the case of, for instance, barley or wheat, to all barley and wheat varieties, including malting barley (e.g., cv Harrington), feed barley (e.g., cv AC Lacombe) and forage barley (e.g., T89043003NX), wheat (e.g., cvs AC Nanda and AC Fielder) and durum wheat amphiploids.

Any plant cells or tissues which remain embryogenic, or which can be induced to return to an embryogenic state, can be used. Such cells or tissues can be induced to form viable plant embryos under appropriate culture conditions. Preferred primary explants include, without limitation, immature embryos, inflorescences, immature inflorescences, and meristems. In cereals, a scutellum isolated from an immature embryo may have a preferred length of 1–2.5 mm, but a length of 1.5–2 mm (about 14 days post-anthesis) is most preferred. A scutellum is a modified leaf, which is the equivalent of the cotyledon in dicots. The scutellum nourishes the germ during embryogenesis and during germination. Monocotyledonous embryos progress through globular, coleoptilar, and scutellar stages (Carman, 1990; Merkle et al., 1995).

Although it is desirable to separate the germ from the scutellum, care should be taken to avoid damaging the scutellum. In order to remove the germ from the embryo, a hook-shaped blade, in which the hook is sized to match the size of the germ, that is which has a radius of curvature to match the outer surface of the germ to be extracted, may advantageously be used. The germ should be removed cleanly, without damaging the scutellum. This prevents the embryo axis (the germ) from interacting with the development of the scutellum cells, thereby avoiding the need for later dissection to remove germinating shoots and roots from the plated embryo.

In other aspects, the invention is useful for inducing embryogenesis in callus cells, suspension cells, and microspore-derived embryos. Callus or calli is a mass of unorganized tissues made up of undifferentiated cells that normally divide very rapidly. Callus is usually induced by the presence of auxin. It also typically forms as a response of tissues to stress, and occasionally develops on a wound in vivo. Suspension cells or suspension cultures comprise substantially homogeneous suspensions of microcalli in liquid medium, whereas a microspore-derived embryo arises from an induced microspore in anther culture or isolated microspore culture. Procedures for obtaining callus cells, suspension cells, or microspore-derived embryos of monocotyledonous plants are known in the art (Lorz et al., 1990; Maheshwari et al., 1995; Bhaskaran et al., 1990).

ii) Tissue Culture Media

Plant tissue culture media typically include substances that can be categorized into seven groups, as follows: salts, sugars, amino acids, hormones (i.e. plant growth regulators), organic acids, vitamins, and gel. Without being bound by same, it is believed that, in the present invention, it is the plant hormone composition of the media that is of greatest significance. In each of the media described herein and in subsequent steps, a wide variety of each of salts, sugars, amino acids, organic acids, and vitamins have been provided in the media to ensure that the media is not deficient in any of these aspects. By and large, for the purposes of the invention, any particular salt, sugar, amino acid, organic acid, or vitamin could be replaced by an alternative, functionally equivalent salt, sugar, amino acid, organic acid, or vitamin, as are known in the art, without a negative effect. Useful basal media include MS (Murashige and Skoog, 1962), and B5 (Gamborg et al., 1968) which are good tissue culture media for most monocot species. George et al. (1987) provide a useful compendium of plant culture media and suitable applications. As salts, sugars, amino acids, organic acids, and vitamins have been included in the media exemplified herein with a view to creating redundancy and excess, one or more of the specified salts, sugars, amino acids, organic acids, or vitamins likely may be reduced in quantity or eliminated, without adverse effect. Tissue culture media may be liquid, solid, or semi-solid. Those skilled in the art can manipulate these aspects of the media formulations and adapt them as needed, within the scope of the invention. All components of plant tissue culture media described herein can be purchased from Sigma-Aldrich, Inc. (St. Louis, Mo., USA).

Tissue culture media used in the invention contain plant growth regulators categorized as auxins, cytokinins, and polyamines. Auxins comprise a family of compounds grouped by function, and which do not share a common chemical structure. Compounds are generally considered to be auxins if they can be characterized by their ability to induce cell elongation in stems and otherwise resemble indoleacetic acid (the first auxin isolated) in physiological activity (Arteca, 1996). Auxins thus may include naturally-occurring auxins such as, without limitation, indole-3-acetic acid (IAA), IAA conjugated with an amino acid, indole 3-butyric acid (IBA), indole 3-butyric acid-potassium salt (KIBA), phenylacetic acid (PAA), and synthetic auxins such as, without limitation, 2,4-dichlorophenoxyacetic acid (2,4-D), dicamba (2-methoxy-3,6-dichlorobenzoic acid), picloram (4-amino-3,5,6-trichloropiconilic acid), 4-chlorophenoxyacetic acid (CPA), α-napthaleneacetic acid (NAA), and β-napthoxyacetic acid (NOA), or combinations thereof. Particularly preferred auxins are 2,4-D and PAA.

Like auxins, cytokinins encompass disparate chemical structures, though many have a structure resembling adenine. Cytokinins promote cell division, and have other functions similar to those of kinetin. Kinetin was the first cytokinin discovered, and was so named because of its ability to promote cytokinesis (i.e. cell division). Though it is a natural compound, kinetin is not produced in plants, and is therefore usually considered a "synthetic" cytokinin (i.e. the hormone is synthesized by an organism other than a plant). The most common form of naturally occurring cytokinin in plants is zeatin, which was isolated from corn (*Zea mays*). Cytokinins have been found in almost all higher plants as well as mosses, fungi, bacteria, and also in tRNA of many prokaryotes and eukaryotes. In excess of 200 natural and synthetic cytokinins are known. Cytokinin concentrations are highest in meristematic regions and areas of continuous growth potential such as roots, young leaves, developing fruits, and seeds (Arteca, 1996). Cytokinins may include naturally occurring cytokinins such as, without limitation, benzyl amino purine (BAP), 6-benzylaminopurine (BA), 6-benylaminopurine riboside (BAR), 6-(γ,γ-dimethylallylamino) purine (2iP), 6-(γ,γ-dimethylallylamino) purine riboside (2iP-R), N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU) zeatin, zeatin riboside (ZR), dihydrozeatin, and thidiazuron, and synthetic cytokinins such as, without limitation kinetin and kinetin riboside (KR), or combinations thereof. A particularly preferred cytokinin is BAP.

There is some controversy as to whether polyamines should be classified as plant hormones. They are widespread in all cells, and they exert regulatory control over growth and development, even at very low levels. Polyamines have a wide range of effects on plants, and they appear to be essential in growth and cell division. Unlike auxins and cytokinins, the polyamines known to function as plant hormones are all related, naturally-occurring compounds found in the same biosynthetic pathway. Such polyamines include, without limitation, the plant growth regulators spermine, spermidine, putrescine, and cadaverine, with spermine and spermidine being particularly preferred.

The plant growth regulators may be present in the media in the form of a single compound within a hormone class (e.g. the auxin component consists only of 2,4-D), or as a combination of compounds (e.g. the auxin component includes both 2,4-D and picloram). Although the compounds within a hormone class are believed to be largely interchangeable, it is believed that the polyamines are an exception, and that spermine and spermidine are functionally distinct from putrescine and cadaverine as plant hormones. Although spermine and spermidine may be used interchangeably in the invention, it is believed that neither putrescine or cadaverine can entirely replace spermine or spermidine in the media. All of the plant growth regulators described herein may be obtained from Sigma-Aldrich, Inc. (St. Louis, Mo., USA).

iii) Direct Somatic Embryogenesis

In one aspect of the invention, the embryogenic monocotyledonous plant cells are first cultured under conditions conducive to direct formation of primary somatic embryos without an intervening callus stage. Somatic embryogenesis is the formation of embryos from vegetative tissues with 2n chromosomes, rather than by sexual reproduction. Somatic embryos originate from small clusters of undifferentiated cells or single cells, and are morphologically analogous to zygotic (fertilized) plant embryos formed through sexual reproduction. Somatic embryos develop normally, and produce plants that are indistinguishable from those obtained from zygotic embryos. Direct somatic embryogenesis describes a form of embryogenesis wherein embryos develop from vegetative cells without an intervening callus step or stage. In contrast, indirect somatic embryogenesis occurs when embryos develop from callus tissues induced in vitro. Elimination of a callus stage through direct somatic embryogenesis advantageously avoids undesirable somaclonal variation. This can be accomplished by cultivating the cells in or on a culture medium which contains plant hormones in concentrations and relative proportions as described herein.

In order to induce direct somatic embryogenesis, the cells are cultured on or in media containing auxin, cytokinin, and polyamine, wherein auxin is present in greater proportion than cytokinin. Preferably, the ratio of auxin to cytokinin in the culture medium is from about 5 μM auxin per 1 μM cytokinin to about 20 μM auxin per 1 μM cytokinin, and is more preferably about 14 μM auxin per 1 μM cytokinin.

In absolute terms, the medium preferably contains: from about 15 μM to about 45 μM auxin; from about 15 μM polyamine to about 45 μM polyamine; and, from about 1 μM cytokinin to about 5 μM cytokinin. More preferably, the culture medium contains: about 30 μM auxin; about 30 μM polyamine; and, about 2 μM cytokinin.

In an exemplified case, the medium is "DSEM" medium, the composition of which is specified in Table 2 herein. Those of ordinary skill in the art will appreciate that the composition of the DSEM medium, particularly with respect to the salts, sugars, amino acids, organic acids, vitamins, and gel, can be modified or adapted without departing from the scope and spirit of the invention.

The cells are not cultured for a pre-determined period of time, but rather at least until, as determined by observation, at least one primary embryo reaches the globular developmental stage. FIG. **1** illustrates an immature barley scutellum at the time of culture initiation, prior to the commencement of embryogenesis. The immature scutellum appears to lack distinct structures. However, FIG. **2** shows the immature scutellum after five days of culture, at which time direct somatic embryogenesis has commenced since numerous primary embryos at the globular developmental stage are observed. These globular embryos **12** may be recognized by their spherical shape on the surface of the scutellum. A small amount of calli **10**, which appears soft and friable, is present on a different part of the scutellum. To assess developmental stage, the tissues may thus be examined for such characteristic features under a stereo-microscope.

Proceeding on the basis of developmental stage, rather than in accordance with a rigid time table, enables the processes of the invention to provide fertile, green plants faster than do prior art techniques. Typically, many primary embryos will reach the globular developmental stage simultaneously, but the developmental rate of all primary embryos will not be identical. It is sufficient that the cells remain on the first culture medium until at least one primary embryo reaches the globular developmental stage. However, if the number of secondary embryos to be obtained in a subsequent step of secondary embryogenesis is to be maximized, and the overall time to obtain fertile plants is of lesser concern, the cells may be cultured longer, up until the point that primary embryos reach the coleoptilar stage. FIG. **2** depicts well the point at which transfer of the globular-stage primary embryos to a culture for secondary embryogenesis is desirable.

iv) Secondary Embryogenesis

A significant advantage of somatic embryogenesis is that it provides an opportunity for manipulating primary embryos such that, rather than proceeding to the next stage in their ontogeny, they instead give rise to new somatic embryos (i.e. recurrent or secondary embryogenesis). The proliferation of embryos can be exploited for mass propagation and the production of transgenic plants. Secondary embryogenesis is, however, an optional step in the invention. There may be occasions where it is instead desired to proceed directly to the step of regeneration of plantlets.

If secondary embryogenesis is desired, in accordance with the invention, the embryogenic cells, in which direct somatic embryogenesis has been induced, are cultured under conditions conducive to induction of secondary embryo formation, for a period of time that persists at least until secondary embryogenesis is detected. The length of the secondary embryogenesis culture step is determined by observation of the cultured tissues, rather than in accordance with a predetermined time line. Although it may be desired to continue culturing secondary embryos for repetitive cycles of embryogenesis, cells may be transferred to the next step once secondary embryogenesis can first be detected with the use of a stereo-microscope. In this respect, FIG. **3** illustrates secondary embryogenesis in wheat. Secondary embryos **14** arise at the equatorial plane of a germinating primary embryo **12**. They initially appear as bulges growing at the surface of the germinating primary embryo, and soon switch

to the coleoptilar stage. The coleoptilar stage is signified by the appearance of primordium of coleoptiles which may appear partly circular, perfectly circular, or more complex in shape and may cover the meristem and first leaf of the embryo. Primary leaves **16** and a small amount of tissue remaining from the scutellum **18** are indicated. Typically, the cells will be cultured under conditions conducive to secondary embryogenesis at least until a first generation of secondary embryos is well-developed. FIG. **3** is a good illustration of the point at which transfer of the secondary embryos to a culture medium conducive to regeneration of plantlets is desirable.

"Primary embryogenesis" refers a first stage or cycle of somatic embryogenesis, in which the embryos which are formed are referred to as "primary embryos." "Secondary embryogenesis" refers to a second stage or cycle of somatic embryogenesis to give rise to new somatic embryos, termed "secondary embryos."

In order to induce secondary embryogenesis, the cells are cultured in or on a solid, semi-solid or liquid medium containing auxin, cytokinin, and polyamine, wherein cytokinin is present in greater proportion than auxin.

Preferably, the ratio of auxin to cytokinin in the culture medium used to induce secondary embryogenesis is from about 0.05 μM auxin per 1 μM cytokinin to about 0.2 μM auxin per 1 μM cytokinin, and is more preferably about 0.1 μM auxin per 1.0 μM cytokinin.

In absolute terms, the secondary enibryogenesis medium preferably contains: from about 5 μM auxin to about 15 μM auxin; from about 15 μM polyamine to about 45 μM polyamine; and, from about 50 μM cytokinin to about 200 μM cytokinin. More preferably, the culture medium contains: about 11 μM auxin; about 30 μM polyamine; and, about 110 μM cytokinin.

In an exemplified case, the medium is "SEM" medium, the composition of which is specified in Table 2 herein. Those of ordinary skill in the art will appreciate that the composition of the SEM medium, particularly with respect to the salts, sugars, amino acids, organic acids, vitamins, and gel, can be modified or adapted without departing from the scope and spirit of the invention.

In another aspect of the invention, there are circumstances wherein the step of secondary embryogenesis may not be needed or desired, and it is possible to proceed directly from direct somatic embryogenesis to the step of germination or regeneration. For example, the processes of the invention are useful in connection with screening procedures, wherein plants are to be tested for tolerance to certain substances, such as salt, or compounds involved in disease conditions (e.g. trichothecenes for fusarium heat blight (FHB) resistance). If the plant cells are transformed with foreign genes, the step of secondary embryogenesis would generally be of benefit for reducing the number of chimeric plants regenerated and obtaining more green and fertile plants. If, however, chimeric plants are not unacceptable, the cells may be transferred directly from the direct somatic embryogenesis culture conditions to germination or regeneration.

v) Organogenesis

Due to variations which naturally occur with somatic embryos and factors such as the species of plant and explant source, cells may have different developmental fates, such that some cells produce embryos (i.e. embryogenesis), while others form shoot or root primordia (i.e. organogenesis). While primary embryos, for example of barley, wheat, oat, rye, and durum wheat as demonstrated in Examples 1, 2 and 3, form secondary embryos under appropriate culture conditions, other species, such as sorghum and corn as reported in Example 4, react differently to the same culture conditions. Organogenesis refers to induction of new organs, such as new adventitious shoots or roots. As reported herein, organogenesis may arise following culturing of globular-stage primary embryos obtained from direct somatic embryogenesis. Organogenesis, as detected by at least the formation of adventitious shoots, may occur in species such as sorghum and corn as demonstrated in Example 4. The adventitious shoots are then cultured to regenerate plantlets. Thus, it appears possible to regenerate plantlets of particular plant species, such as sorghum and corn genotypes as reported herein, through the processes of direct somatic embryogenesis and organogenesis.

The embryogenic monocotyledonous plant cells are first cultured under conditions conducive to direct formation of primary somatic embryos without an intervening callus stage, as described previously. Briefly, the cells are cultured on or in media containing auxin, cytokinin, and polyamine, wherein auxin is present in greater proportion than cytokinin.

Preferably, the ratio of auxin to cytokinin in the culture medium is from about 5 μM auxin per 1 μM cytokinin to about 20 μM auxin per 1 μM cytokinin, and is more preferably about 14 μM auxin per 1 μM cytokinin.

In absolute terms, the medium preferably contains: from about 15 μM to about 45 μM auxin; from about 15 μM polyamine to about 45 μM polyamine; and, from about 1 μM cytokinin to about 5 μM cytokinin. More preferably, the culture medium contains: about 30 μM auxin; about 30 μM polyamine; and, about 2 μM cytokinin.

In an exemplified case, the medium is "DSEM" medium, the composition of which is specified in Table 2 herein. Those of ordinary skill in the art will appreciate that the composition of the DSEM medium, particularly with respect to the salts, sugars, amino acids, organic acids, vitamins, and gel, can be modified or adapted without departing from the scope and spirit of the invention.

In order to induce organogenesis, the globular-stage primary embryos are then cultured on or in media containing auxin, cytokinin, and polyamine, wherein cytokinin is present in greater proportion than auxin.

Preferably, the ratio of auxin to cytokinin in the culture medium is from about 0.05 μM auxin per 1 μM cytokinin to about 0.2 μM auxin per 1 μM cytokinin, and is more preferably about 0.1 μM auxin per 1.0 μM cytokinin.

In absolute terms, the medium preferably contains: from about 5 μM to about 15 μM auxin; from about 15 μM polyamine to about 45 μM polyamine; and, from about 50 μM cytokinin to about 200 μM cytokinin. More preferably, the culture medium contains: about 11 μM auxin; about 30 μM polyamine; and, about 110 μM cytokinin.

In an exemplified case, the medium is "SEM" medium, the composition of which is specified in Table 2 herein. Those skilled in the art will appreciate that the composition of the SEM medium, particularly with respect to the salts, sugars, amino acids, organic acids, vitamins, and gel, can be modified or adapted without departing from the scope and spirit of the invention.

The globular-stage primary embryos are cultured until induction of organogenesis or the formation of new organs, as detected by the presence of adventitious shoots. The new shoots obtained are cultured in or on regeneration medium to produce plantlets. Preferably, the medium is MS_{reg}, the composition of which is set forth in Table 2 herein. The plantlets and shoots are cultured under conditions conducive

to root formation, after which the plantlets are transplanted to soil and grown to maturity.

vi) Induction of Embryogenesis in Embryogenic Monocotyledonous Callus Cells, Suspension Cells, or Microspore-derived Embryos

The foregoing aspects of the invention are directed principally to inducing direct somatic embryogenesis in primary explants, such as immature embryos, meristems, and inflorescenses. However, aspects of the invention may also be used for rapidly inducing embryogenesis in embryogenic monocotyledonous callus cells, suspension cells, or microspore-derived embryos.

In yet another aspect of the invention, when working with callus cultures or suspension cells or microspore-derived embryos, the step of direct somatic embryogenesis is eliminated. The monocotyledonous plant cells of interest are cultured under SEM medium followed by a germination or regeneration step. This approach is useful as an alternative to the standard two-step induction and regeneration process. Embryogenesis is induced by culturing the cells in or on a medium containing auxin, cytokinin, and polyamine, wherein cytokinin is present in greater proportion than auxin, as described above in this section. Preferred media compositions are also as described hereinbefore. If the cells of interest are callus or suspension cells, the resulting embryogenesis cannot properly be characterized as primary or secondary embryogenesis, but merely as embryogenesis. The cells may be transferred to the regeneration step once embryogenesis is detected. Preferably, however, the cells are not transferred to the next step until embryogenesis is substantially complete. If microspore-derived embryos are used as the starting material, the result is secondary embryogenesis, much as if the tissues had been transferred from the step of direct somatic embryogenesis.

vii) Germination

In some instances, it may be advantageous, after the step of secondary embryogenesis (or, if secondary embryogenesis is excluded -then after the step of direct somatic embryogenesis) to cultivate the cells under conditions conducive to germination of the primary or secondary embryos. For this purpose, a solid, semi-solid or liquid culture medium containing polyamine in amount effective to cause germination of the embryos, and which is essentially free of either auxin or cytokinin, is preferred. Preferably, the germination medium contains from about 15 μM polyamine to about 45 μM polyamine, more preferably about 30 μM polyamine. In a preferred embodiment, the germination medium is "GEM" medium, the composition of which is described in Table 2 herein. It will be understood that the composition of the GEM media, apart from the plant hormone component, is not essential, and that it may be adapted or optimized to meet the requirements of particular circumstances without departing from the invention.

Cells are cultured on the germination medium until germination of at least one embryo commences. Germination is the growth of leaves and roots from the germ. Preferably, the developing embryos are cultured until the germination has commenced in a majority of embryos, though a lesser degree of germination may be sufficient. Once a satisfactory degree of germination has been established, the cells can be transferred to the regeneration step.

viii) Regeneration

In order to produce plantlets, the developing embryos are preferably cultured in or on a regeneration medium. A variety of suitable regeneration media are known in the art as described by George et al. (1987). The choice of regeneration medium is not critical. A particularly suitable regeneration medium is MS_{reg} (Murashige and Skoog, 1962), the composition of which is set forth in Table 2 herein. The low auxin concentration of MS_{reg} favours the formation of green shoots from primary and secondary embryos.

Root initiation and development in wheat tissues often occurs during the regeneration step. In that case, once root formation is established, the developing plantlets can be directly transferred to pots containing potting mix. The choice of potting mix is not critical, and many are known in the art. For instance, a suitable potting mix is Cornell mix.

There may be instances wherein root development does not initiate during the regeneration step. This is often the case with barley tissues, for example. If roots do not develop on regeneration media, a mass of cells forming green shoots is placed on a last medium for root initiation. Suitable rooting media include MS, or a half dose of MS (Ahloowalia, 1982), the rooting medium of Green (1982), or rooting medium R of Schaeffer et al. (1984). Once roots have been established, plantlets are transferred to potting mix.

ix) Transformation

The processes of the invention are particularly useful in connection with the introduction of foreign genes or DNA into monocot plant cells. Foreign genes which may be introduced into plants in accordance with the invention include, without limitation, genes related to quality traits, disease or pathogen resistance genes, stress resistance genes, herbicide resistance genes, and genes which are introduced into plants for large-scale recovery of the encoded product by molecular farming.

Genes relating to quality traits include, for instance, β-glucanase genes (e.g. egl1), which have been introduced into barley plants in order to improve malting (Mannonen, 1993). Genes encoding other enzymes, such as xylanases, or phytases, may be introduced into plants to increase their digestibility or phosphate absorption when used as animal feeds. An interesting example of a quality trait introduced into a cereal crop plant is the introduction into rice of a gene encoding the last enzyme in the pathway for vitamin A biosynthesis (Burkhardt et al., 1997). The resulting transgenic rice may be beneficial in the Third World for preventing blindness in children, a condition which often results from vitamin A deficiency.

A number of disease resistance genes have been introduced into plants, and may be used in connection with the invention. Examples include the barley yellow dwarf virus resistance gene (BYDV) (Wan and Lemaux, 1994), the thaumatin fungal resistance gene (Rogers and Rogers, 1992), and the stripe rust resistance gene (Yr10), which is exemplified in the Examples herein. Other disease resistance genes which may be introduced into plants in accordance with the invention are known in the art, a number of which are enumerated by Mannonen et al. (1994). Alternatively, the foreign gene may confer resistance to insects or other pests. Such genes include, without limitation, those that encode enzyme inhibitors, insect-specific hormones or pheromones, insect-specific peptides or neuropeptides which disrupt the physiology of the targeted pest, or an insect-specific poison or venom produced in nature.

Herbicide resistance genes are known in the art, and may be introduced into plants in accordance with the processes of the invention. Examples include: CP4, a bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene which expresses an enzyme highly resistant to glyphosate, the active ingredient in the herbicide ROUND-UP™;

the glyphosate oxidoreductase (GOX) gene, which is a bacterial gene which degrades glyphosate into aminomethyl phosphoric acid (U.S. Pat. No. 5,631,152 to Fry et al.); and the bar gene, which provides resistance to glufosinate ammonium, the active ingredient in LIBERTY™ herbicide.

Genes which confer stress resistance properties may be introduced into monocotyledonous plants in accordance with the invention. Desirable stress-resistance properties include, without limitation, salt tolerance (Lee et al., 1999), drought tolerance (Sheveleva et al., 1997), cold tolerance (Hayashi et al., 1997), and heavy metal tolerance, such as aluminum tolerance (De La Fuente et al., 1997)

Involving the use of transgenic plants to produce large quantities of a desired gene product, molecular farming has many advantages over traditional microbial fermentation systems, including elimination of the requirements for extrinsic energy sources, sophisticated fermentation apparatus, and closely controlled culture conditions. Plants transformed in accordance with the invention may be used for such disparate purposes as making therapeutic proteins, enzymes, antibodies, vaccines, or making biodegradable plastics (Fischer et al., 1999; Elliott et al., 1996; Hemming, 1995; Pen et al., 1993).

Vectors for introducing foreign DNA into plant cells are well known in the art (Gruber et al. (1993). Suitable recombinant vectors include an expression cassette designed for initiating transcription of the foreign DNA in plants. Additional sequences can be included to allow the vector to be cloned in a bacterial or phage host. The vector will preferably contain a prokaryote origin of replication having a broad host range. A selectable marker may also be included to allow selection of bacterial cells bearing the desired construct. Suitable prokaryotic selectable markers include those that confer resistance to antibiotics such as ampicillin. Other DNA sequences encoding additional functions may also be present in the vector. For instance, in the case of *Agrobacterium*-mediated transformation, T-DNA sequences will also be included for subsequent transfer to plant chromosomes.

For expression in plants, the recombinant expression cassette preferably contains, in addition to the desired gene sequence to be expressed, a promoter region effective in plants, a transcription initiation site (if the sequence to be transcribed lacks one), and a transcription termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette are typically included to allow for easy insertion into a pre-existing vector. Sequences controlling eukaryotic gene expression are well known in the art.

The particular promoter used in the expression cassette is not critical to the invention. Any of a number of promoters which direct transcription in monocotyledonous plant cells is suitable. The promoter can be either constitutive, inducible, tissue specific, or temporal specific. A number of promoters which are active in plant cells have been described in the literature, including the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumour-inducing plasmids of *Agrobacterium tumefaciens*), the caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S and the figwort mosaic virus 35S-promoters, the maize adh1 promoter, the light-inducible promoter from the small subunit of ribulose-1,5-bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide), and the chlorophyll a/b binding protein gene promoter, and a cryptic promoter (tCUP) from tobacco. All of these promoters have been used to create various types of DNA constructs which have been expressed in plants. Preferred promoters include: the actin promoter, a constitutive promoter from rice (McElroy et al., 1990); *Ubi*-1, a constitutive promoter functional in many monocots (Wan and Lemaux, 1994); *Hordein B1*, a seed specific promoter from barley (Knudsen and Müller, 1991); HMW glutelin (Lee et al., 1991), a tissue-specific promoter from wheat; and *α-amy-I*, a tissue-specific promoter from wheat (Jacobsen and Close, 1991).

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes. A useful transcription termination region is the nopaline synthase NOS 3' terminator sequence (Bevan et al. 1983).

Polyadenylation is believed to have an effect on stabilizing mRNAs. Therefore, polyadenylation sequences are also commonly added to the vector construct if the mRNA encoded by the structural gene is to be efficiently translated (Alber and Kawasaki, 1982). Polyadenylation sequences include, but are not limited to the *Agrobacterium* octopine synthase signal (Gielen et al., 1984) or the nopaline synthase signal (Depicker et al., 1982). The vector will also typically contain a selectable marker gene by which transformed plant cells can be identified in culture. Numerous selectable marker genes are known in the art and are readily available. Typically, the marker gene encodes antibiotic resistance or herbicide resistance. These markers include those that confer resistance to the antibiotics ampicillin (the amp gene), kanamycin (the nptil gene), chloramphenicol (the cat gene), G418, hygromycin, bleomycin, kanamycin, and gentamycin. Other markers confer resistance to herbicides, such as the bar gene, which confers resistance to glufosinate ammonium, the active ingredient of the herbicides BIOLAPHOS™, BASTA™ and LIBERTY™. Those cells containing the vector will be identified by their ability to grow in a medium containing the particular selective agent.

The vector may also contain a reporter gene for the analysis of plant gene expression. A suitable reporter gene is the bacterial gene uidA, encoding β-glucuronidase (GUS). GUS expression can be conveniently quantified through a highly sensitive non-radioactive assay using the fluorogenic substrate 4-methylumbelliferyl glucuronide (MUG) (Spoerlein et al., 1991). Other suitable reporter genes include those encoding beta-galactosidase, luciferase, and chloramphenicol acyltransferase.

Once an appropriate vector has been assembled, a variety of techniques are available for introducing the vector into the monocotyledonous plant cells (Potrykus, 1990). Although *Agrobacterium*-mediated transformation is used principally in dicot species, recent evidence suggests that monocots could be transformed with hypervirulent *Agrobacterium* strains (Creissen et al., 1990). DNA may also be transferred to monocot protoplasts either by electroporation (Fromm et al., 1986) or through the use of polyethylene glycol (PEG) (Paszkowski et al. 1984). Alternatively, the vector can be micro-injected directly into plant cells (Toyoda et al., 1990) or introduced into cells electrophoretically (Ahokas, 1989). Other approaches to transforming plant cells include fusion of cereal protoplasts with cationic liposomes containing DNA (Antonelli and Stadler, 1990), penetration of the cell wall with a laser beam (Kaneko et al., 1991), and dry seed imbibition in DNA solution (Töpfer et al., 1989).

In accordance with the invention, particle bombardment (Weeks et al., 1993; Wan and Lemaux, 1994; Cho et al., 1998) is a preferred method for introducing the vector into

the monocotyledonous plant cells. In this technique, DNA coated microcarriers are accelerated to high velocity by a particle gun apparatus. Due to acceleration, the microcarriers cross the cell wall/membrane barrier, deliver the foreign DNA inside the cell, and the transformants are regenerated under selection. The microcarriers should be of sufficient mass to possess adequate momentum to penetrate the appropriate tissue. Suitable metal particles include gold, tungsten, palladium, rhodium, platinum, iridium and perhaps other second and third row transition metals. Metals should be chemically inert to prevent adverse reactions with the DNA or cell components The particles may be selected for size and shape, as well as agglomeration and dispersion properties, as are known in the art. Certain additives such as spermidine and calcium chloride may be beneficially added to the DNA coated onto the microcarriers. Suitable particle gun apparatus is known in the art and is readily available, such as the Helios Gene Gun System (Bio-Rad, Hercules, Calif.) which is suitable. Baffles or mesh screens may be used to reduce physical trauma to cells from the gas blast and acoustic shock generated by the particle gun, resulting in reduced cell death and increased transformation efficiency.

Transformation of plant cells, particularly by particle bombardment or *Agrobacterium*-mediated transformation, is preferably conducted prior to development of the primary embryo under conditions conducive to direct somatic embryogenesis, but may occur anywhere from about zero to five days after commencement of tissue culture. Prior to transformation, the culture medium does not contain a selective agent. Approximately 16 hours after transformation, the cells are preferably transferred to media supplemented with the appropriate selective agent. For instance, if the marker gene used is the bar gene, an appropriate selective agent is glufosinate ammonium. The selective agent preferably is present during each of the subsequent tissue culture and regeneration steps. If the step of direct somatic embryogenesis is not included, transformation of callus cells, suspension cells, or microspore-derived embryos may be effected prior to culturing on SEM medium.

After transformation, transformed plant cells or plants carrying the introduced DNA are identified, typically by selection for a marker gene and/or a reporter gene, as described previously.

In an exemplified case, barley cells transformed with the bar marker gene were selected by growing the cells on growth medium containing glufosinate ammonium. Only successfully transformed cells that were resistant to the herbicide survived. The presence of the uidA reporter gene in the surviving cells was detected by the observation of blue dots on one of the regenerated plants, indicative of β-glucuronidase (GUS) activity.

Alternatively, expression of the foreign DNA can be confirmed by detection of RNA encoded by the inserted DNA using well known methods such as Northern blot hybridization. The inserted DNA sequence can itself be identified by Southern blot hybridization or the polymerase chain reaction (PCR) (Sambrook et al., 1989; Ausubel et al., 2000). In Examples 5 and 6 herein, the presence of the bar and uidA genes was confirmed by PCR amplification and Southern blot hybridization (Tables 7 and 8).

Recombinant DNA procedures and tissue culture procedures used for practicing the invention and which are not described in detail herein involve standard laboratory techniques as described in Sambrook et al. (1989), Ausubel et al. (2000), or Zryd and Richards (1988).

Generally, enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. Abbreviations and nomenclature employed herein are standard in the art and are commonly used in scientific publications such as those cited herein.

The invention is further illustrated by the following non-limiting Examples.

EXAMPLE 1

Direct Somatic Embryogenesis, Secondary Embryogenesis and Regeneration of Barley Genotypes

i) Explant Source

Immature embryos of seven barley genotypes, as described in Table 3 herein, were used for this study, including Canadian six-row forage lines, two-row feed barley varieties and the two-row malting barley cultivar Golden Promise. Golden Promise is used extensively as a model cultivar for barley because of its ability to regenerate relatively well with in vitro culture protocols (Bregitzer et al., 1998).

Stock plants were grown in growth chambers with 16-h photoperiod (bottom/top of plants: 270/330 μmol m^{-2} s^{-1}) with 21/16° C. day/night temperature regimes. Immature spikes were harvested around 14 days post-anthesis. At this developmental stage, the embryos varied from 1.5 to 2 mm in size. Spikes were collected and kept in a refrigerator at 4° C. for three to seven days for a cold pre-treatment. Immature caryopses were collected and then washed for 30 s with 70% ethanol, and surface sterilized in 10% bleach for 10 min in a laminar flow hood. Then, three washes with double distilled sterilized water were applied for 1 min each.

Dissection of the caryopses was carried out under a stereo-microscope, using forceps and a modified scalpel blade (shaped over a burner to form a hook having a radius of curvature to match the outer surface of the germ to be extracted) to dissect the immature seed. The caryopsis was held with the forceps, and the seed coat above the immature embryo was removed with the blade. The exposed germ was then cut out, and the scutellum gently removed from the caryopsis with the smooth side of the blade. The scutellum was placed up-side-down on the first medium, DSEM (Table 2). This approach minimized physical damage to the scutella, ensured that all germ tissues were removed, and accelerated the isolation of scutella.

ii) Immature Scutella Culture

Excised scutella were grown in the dark at 25° C. on DSEM medium (composition of all media is reported in Table 2), until primary embryos located on the top of scutella were developed to the globular developmental stage. Tissues were examined under a stereo-microscope to determine the developmental stage. Tissues at the globular developmental stage were transferred to the SEM medium, and cultivated in the dark at 25° C. until secondary embryogenesis could be detected using a stereo-microscope. Then, tissues carrying primary and secondary embryos were cut into two to four pieces and transferred to GEM medium. Most of the embryos grew, and some could germinate on GEM medium. When two or more secondary embryos were attached to a primary embryo, these tissues were preferably cut again, before putting them in the regeneration medium MS_{reg}, under light (80 μmolm^{-2} s^{-1}) at 16° C. To further increase the number of regenerated plants, all primary embryos were

systematically cut to favor the germination of all secondary embryos. Green embryos that germinated on the regeneration medium were removed and transferred to a rooting medium in Magenta boxes, under the same light conditions, which allowed plantlets to develop their roots.

iii) Experimental Method

Twenty scutella were randomly plated in each petri dish. Each genotype was represented by three dishes containing 20 scutella each. For each experimental unit, the number of scutella that produced plantlets and the total number of plantlets were recorded. A "plantlet" was defined as a small regenerated plant with green shoots and roots that arose from either germinating primary or secondary embryos. Mean and standard deviation for the mean were calculated for each genotype.

iv) Results

The same developmental pattern for direct somatic embryogenesis was observed on scutella of the seven barley genotypes representing forage, feed and malting barley. Direct somatic embryogenesis up to the globular stage was induced by the DSEM medium. Globular embryos were observed on most scutella. Two forage type barley lines, H84108005NX and H8902001N, produced as many direct somatic embryos as Golden Promise, a non-recalcitrant barley cultivar for tissue culture (Table 3). Secondary embryogenesis was induced by the SEM medium from the primary globular embryos for all genotypes tested. The secondary embryos always developed from the primary globular embryos, which sometimes started to germinate on the SEM medium. Further development of the primary embryos occurred in the GEM medium when the number of embryos were low. It appeared that the withdrawal of cytokinin partially enabled the larger primary embryos to inhibit the growth of the younger secondary embryos, which is similar to the apical dominance observed in adult plants. Cutting scutella to allow primary embryos to develop freely was beneficial, but it was not possible without damaging the primary embryos. On average, 50 plantlets from germinating primary and secondary embryos were regenerated from the initial 20 scutella. Forage and feed barley cultivars had been previously identified as recalcitrant to in vitro culture regeneration. All lines but one yielded numbers of plantlets similar to Golden Promise (Table 3). The number of plants regenerated was impressive despite the fact that the quality of the stock plants used for isolating the scutella were poor due to disease infection. Stress generally reduced the ability of plants to regenerate in vitro. A preferred method to further increase the number of regenerated plants would be to systematically cut all primary embryos to favor the development and germination of all secondary embryos. These results are also believed to represent the first report of direct somatic embryogenesis and secondary embryogenesis in monocots.

EXAMPLE 2

Direct Somatic Embryogenesis and Secondary Embryogenesis from Scutella of Barley, Wheat and Durum Wheat Amphiploids

i) Immature Scutella Culture

The tissue culture protocols and experimental unit for barley and wheat were essentially the same, and were similar to that described in Example 1. The barley and wheat embryos were 2 mm in size. Tissues were grown in the DSEM medium until the primary embryos on top of scutella were at the globular stage, and then scutella were cut into two pieces.

ii) Results

A very large number of primary and secondary embryos were produced during this experiment. Therefore, only the number of plantlets which germinated from either primary or secondary embryos was recorded (Table 4). Scutella from all barley genotypes followed the sequence of direct somatic embryogenesis, secondary embryogenesis, germination, regeneration and rooting, as in Example 1. The step of growing the embryos on rooting medium was not necessary for the wheat cultivars, because they produced roots easily on the regeneration medium MS_{reg}. One group of genotypes, AC Nanda, AC Fielder, T89037005X, AC Lacombe, Golden Promise and T89047103NX, produced between six and ten primary globular embryos per scutellum. The three other genotypes, T89034001, Harrington and H84107004N, produced about three primary globular embryos per scutellum. Once primary globular embryos were well developed, no differences were observed among genotypes in the numbers of secondary embryos produced.

The secondary embryos were more compact in barley (FIGS. **1** and **2**) than in wheat in which they arise as distinct bulges (FIG. **3**), making identification of barley secondary embryos more difficult. Wheat secondary embryos developed individually in the equatorial plane of the primary globular embryo (FIG. **3**). They were very similar to the zygotic embryos.

Non-stressed plants were used in this experiment, and scutella were similar in size. A uniform response from scutella and a higher number of primary globular embryos were observed in this experiment compared to the results reported in Example 1 (Table 3). Fully developed primary globular embryos responded very well to SEM medium as they produced between one and ten secondary embryos. Preferably, primary globular embryo are fully developed on the DSEM medium in order to get a maximum induction of secondary embryos on the second medium. The scutella were cut in pieces to allow primary embryos to develop freely and to ensure successful germination. However, it was not possible to detach the secondary embryos from the primary embryos without damaging them.

These results showed that the invention is capable of inducing direct somatic embryogenesis and secondary embryogenesis in a wide range of barley and wheat genotypes. The results also demonstrate that direct somatic embryogenesis reduces the number of days required to regenerate plants using in vitro culture, the methods of the invention providing green, fertile barley plants about two months earlier than the typical callus induction and regeneration approach, and green, fertile wheat plants about one month earlier than the best callus induction and regeneration approach. The forage, feed and some malting barley cultivars used in these experiments were previously considered recalcitrant to in vitro regeneration. These results are also believed to represent the first report of direct somatic embryogenesis and secondary embryogenesis in monocots.

Similar results were obtained from the interspecific cross *Triticum durum* cv Calvin/*Elytrigia disticha*//Calvin, BC_1F_5. *Elytrigia disticha* is a grass and a wild relative of wheat. Many disease resistance genes from wild and related grasses have been introduced to wheat. Ten scutella were excised and treated as above. After seven days of culture on the DSEM medium, fully developed primary globular embryos were identified. After another 11 days in SEM medium,

secondary embryogenesis was completed. From the 10 scutella originally plated, 120 plantlets (1200%) were regenerated. These results demonstrate that direct somatic embryogenesis can be used with different species of wheat, *Triticum durum*, and their wild grass relatives.

EXAMPLE 3

Direct Somatic Embryogenesis, Secondary Embryogenesis and Regeneration of Oat, Rye Wheat, Barley, Durum Wheat, *Triticum monococum* and *Triticum urartu*

i) Explant Source

Immature embryos of oat, rye, wheat, barley, durum wheat, *T monococum* and *T. urartu*, as described in Table 5 herein, were used for this study, including the lines of Juniper and CDC Pacer in oat; perennial cereal rye (PC Rye); Hy366-BL31 and P8810-B5B3A2A2 in common wheat; DT701 in durum wheat; accessions 89, 173, 238 in *T. monococum*; accession 17111 in *T. urartu*; Golden Promise and T89047103NX in barley.

Scutella were isolated from the immature embryos as described in Example 1. Briefly, caryopses containing immature embryos at 15 days post-anthesis were dissected. At this developmental stage, the embryos were about 2 mm in length for all species, except those of *T. monococum* and *T. urartu* which were 1.5 mm in length.

ii) Immature Scutella Culture

Excised scutella were placed upside-down on the DSEM medium and grown, until the primary embryos located on top of the scutella reached the globular stage. Direct somatic embryogenesis along the scutellum was observed in all species, with the exception of oat in which direct somatic embryogenesis was observed only at one extremity of the scutellum, while all other parts of the scutellum turned brown and died in a few days. Scutella carrying primary embryos at the globular stage were cut into pieces to separate the primary embryos which were then transferred to SEM medium, on which most of the primary embryos grew, with some germinating early.

iii) Experimental Method

Twenty scutella were randomly plated in each petri dish. Each genotype was represented by three dishes containing 20 scutella each. For each experimental unit, the number of scutella that produced plantlets and the total number of plantlets were recorded. A "plantlet" was observed as a small regenerated plant with green shoots and roots that arose from either germinating primary or secondary embryos.

iv) Results

Most of the genotypes showed a high embryogenic response, while only CDC Pacer (oat) showed a low response. Green and fertile plantlets were regenerated from all genotypes (Table 5). The regeneration was very high in common wheat and barley. A very good level of regeneration was observed for the diploid wheat species *T. monococum* and *T. urartu*, durum wheat and oat.

Regeneration was more modest for one cultivar of oat and the perennial cereal rye. However, regeneration in these species is intriguing, since they are characterized by cross-fertilization and large variation in the temporal developmental stage of embryos. Since an oat panicle has caryopses that vary in age, it was thus more difficult to collect embryos of the same age, and to obtain enough embryos with a suitable size in the same day. Furthermore, under stress, oat embryos produce phenolic compounds that are toxic to the embryo itself. Durum wheat regeneration is significant, in that this species has been recognized as recalcitrant for regeneration. *T. monococum* is also very recalcitrant, although regenerated plantlets were obtained at a high frequency despite the smaller size of the embryo in this diploid wheat. These results are believed to represent the first report of regeneration of the wheat diploid species *T. urartu*.

All genotypes from seven different species were thus regenerated, with common wheat and Golden Promise (barley) showing the highest response, most likely since this novel method was originally designed for barley and common wheat, and the selected size of the scutellum was optimum for such species. Further, the regeneration of these seven species demonstrates that the method is effective for different species, even those (e.g. oat, *Triticum monococum* and *T. urartu*) which have been previously recognized to be recalcitrant to in vitro regeneration, and successfully induces direct somatic embryogenesis and secondary embryogenesis in a wide range of monocotyledonous species. These results are also believed to represent the first report of successful direct somatic embryogenesis and secondary embryogenesis in these seven species of monocots.

EXAMPLE 4

Direct Somatic Embryogenesis from Immature Scutella of Sorghum and Corn Followed by Organogenesis.

i) Explant Source

Immature embryos of sorghum and corn as described in Table 6 herein, were used for this study, including the lines of CK60 and PI229828 in sorghum and H96F and HFDM in corn. Scutella were isolated from the immature embryos as described in Example 1. Briefly, caryopses containing immature embryos at 15 days post-anthesis were dissected. At this developmental stage, the embryos were approximately 2 mm in length.

ii) Immature Scutella Culture

Excised scutella were placed upside-down on the DSEM medium and grown, until the primary embryos located on top of the scutella reached the globular stage. Direct somatic embryogenesis along the scutella was observed in both sorghum and corn. Scutella carrying primary embryos at the globular stage were cut in two pieces to separate groups of primary embryos which were then transferred to SEM medium, on which most of the primary embryos grew.

iii) Experimental Method

Twenty scutella were randomly plated in each petri dish. Each genotype was represented by one dish containing 20 scutella each. For each experimental unit, the number of scutella that produced plantlets and the total number of plantlets were recorded. A "plantlet" was observed as a small regenerated plant with green shoots and roots.

iv) Results

For sorghum, a very large number of primary embryos on DSEM and new shoots on SEM (organogenesis) developed during this experiment; thus, only the number of regenerated plantlets were recorded (Table 6). Sorghum scutella reacted rapidly to the stresses of dissection and in vitro culture. Frequently, one section of the scutellum turned brown and died, while the other carried many globular embryos. A few albino plantlets were regenerated from genotype PI229828, replicate 1, but are not counted in Table 6.

Corn produced fewer primary embryos on DSEM and consequently fewer new shoots on SEM than sorghum.

Scutella from all sorghum and corn genotypes followed the sequence comprising direct somatic embryogenesis (on DSEM), early germination (on SEM), initiation of organogenesis (on SEM), regeneration (on GEM and MS regeneration) and rooting (on rooting medium). Organogenesis, the formation of new shoots, occurred on the stems of early germinated primary embryos. The origin of these shoots is thus very different from that of secondary embryos, which originate at the equatorial plane of primary wheat globular embryos.

These results demonstrate that this novel method is suitable to induce direct somatic embryogenesis and organogenesis for sorghum and corn genotypes. These results are also believed to represent the first report of this combination of developmental patterns in monocots.

EXAMPLE 5

Biolistic Transformation of Barley with the bar and uidA Genes

i) Co-transformation

DNA delivery to the tissues was carried out with the Helios Gene Gun System, a microprojectile bombardment device from Bio-Rad (#165-2431, 2000 Alfred Nobel Drive Hercules, Calif. USA 94547). The plasmid pMB2 (Steve Holzberg, Berkeley University Albany Calif. 94710), carrying the marker gene bar (FIG. 4), and the plasmid pACT-1D (Ray Wu, Cornell University), carrying the reporter gene uidA (FIG. 4), were used. Equal amounts of these two plasmids (25 μg each) were mixed and coated on 25 mg of 1 μm gold particles and distributed on the inside wall of a 60-cm GoldCoat tube according to the manufacturer's instructions. Discharge pressure was set at 120–140 psi and a diffusion screen (#165-2475) was used to ensure an even distribution of the gold particles carrying the plasmids.

The tissue culture protocol was essentially the same as that described in Example 2. Barley scutella were bombarded after two days of culture in the DSEM medium. Twenty scutella were transferred onto a modified DSEM medium supplemented with 0.3M mannitol, from 4 h before bombardment to 16 h after bombardment. Forty scutella of Golden Promise, 40 scutella of H89108005NX, and 20 scutella of Seebe were bombarded with plasmid-coated gold particles.

ii) Regeneration of Glufosinate-tolerant Plants

The scutella were transferred to the DSEM medium containing 5 mg/L of glufosinate ammonium salt (#C140300, Crescent Chemical, Hauppauge N.Y. USA), 16h after bombardment. All other steps of tissue culture were accomplished with the selecting chemical at the above concentration. Embryogenesis was slightly delayed and less frequent during the selection procedure using the glufosinate, but there was no impact on the quality of primary and secondary embryos produced. Only a very few escapes (four plants out of 200) were observed from the tissues bombarded with empty gold particles and non-bombarded tissues. Growth of these negative control tissues was much slower and necrosis was frequent. This confirmed that the selection procedure using glufosinate was very effective to eliminate most of the non-transgenic plantlets.

At the regeneration step, cuttings were done where necessary to isolate tolerant germinating secondary embryos, or groups of secondary embryos, as glufosinate-sensitive germinating embryos turned yellow. Rooting medium was not supplemented with glufosinate ammonium salt. Herbicide-tolerant plantlets were recovered and transferred to potting mix within two to three months of initiation of direct embryogenesis. In contrast, previous methods take more than four months to regenerate recalcitrant genotypes such as Harrington (Cho et al., 1998).

iii) Characterization of Selected Green Plants

Herbicide-resistant regenerated plants were tested with the leaf brush technique (LBT) wherein glufosinate ammonium salt at a concentration of 500 mg/L was applied abundantly to leaves of the regenerated plants with a small paint brush. Polymerase chain reaction (PCR) product was probed to confirm presence of the bar DNA in the plant cells. PCR and Southern blot analyses were used to check reporter gene insertion. Marker (bar) and reporter (uidA) genes were detected in the regenerated plants (Table 7). From a total of 100 bombarded embryos of three genotypes, Golden Promise, H89108005NX and Seebe, nine fertile plants were regenerated. These plants subsequently produced seeds. All plants carried the bar gene, but two did not express a high tolerance to the glufosinate ammonium using the LBT assay. Five plants also carried the uidA gene, and blue dots resulting from the in vivo GUS activity were detected in one plant.

These results confirmed that the selection procedure eliminated the non-transgenic plantlets. The transformation protocol was very efficient with the tissue culture techniques of the invention. Regeneration rate of transgenic fertile plant reached 17% for Golden Promise and 5% for the forage barley H89108005NX. The co-transformation technique resulted in 50% reporter/marker gene insertion ratio for the cultivar/lines. Regeneration of transgenic lines from Seebe was not successful in this particular trial. This may have been due to the low number of scutella used for this cultivar.

EXAMPLE 6

Biolistic Transformation of Barley with bar and gfp Genes

i) Co-transformation

DNA delivery to the tissues was carried out with the Helios Gene Gun System, described in Example 3 above. The plasmid pMB2 (Steve Holzberg, Berkeley University Albany Calif. 94710), carrying the marker gene bar (FIG. 4), and the plasmid pBIN m-gfp5-ER (Jim Haseloff, MRC Cambridge England CB2 2QH), carrying the reporter gene gfp (FIG. 4), were used. Equal amounts of these two plasmids (25 μg each) were mixed and coated on 25 mg of 1 μm gold particles and distributed on the inside wall of a 60-cm GoldCoat tube according to the manufacturer's instructions. Discharge pressure was set at 120–140 psi and a diffusion screen (#165-2475) was used to ensure an even distribution of the gold particles carrying the plasmids.

The tissue culture protocol was essentially the same as that described in Example 2. Barley scutella were bombarded after two days of culture on DSEM medium. Twenty scutella were transferred onto a modified DSEM medium supplemented with 0.3M mannitol, from 4 h before bombardment to 16 h after bombardment. A total of 320 scutella from seven genotypes were bombarded for this experiment.

ii) Regeneration of Glufosinate-Tolerant Plants

The scutella were transferred to DSEM medium containing 5 mg/L of glufosinate ammonium salt (#C140300, Crescent Chemical, Hauppauge N.Y. USA), 16 h after bombardment. The rest of the tissue culture steps were

accomplished with this chemical at the same concentration. Embryogenesis was slightly delayed and less frequent during selection procedure using the glufosinate, but there was no impact on the quality of primary and secondary embryos produced.

At the regeneration step, cuttings were done where necessary to isolate tolerant germinating secondary embryos or groups of secondary embryos, as herbicide sensitive germinating embryos turned yellow and thus were easy to identify. Rooting medium was not supplemented with glufosinate ammonium salt. Herbicide-tolerant plantlets were recovered and transferred to potting mix within two to three months of test initiation. In contrast, previous methods take more than four months to regenerate recalcitrant genotypes such as Harrington (Cho et al., 1998).

iii) Characterization of Selected Green Plants

Herbicide-resistant regenerated plants were tested with the leaf brush technique as in the previous Example. PCR product was probed to confirm presence of the bar DNA into the plant cells. PCR and Southern blot technique were used to check reporter gene insertion. Marker (bar) and reporter (gfp) genes were detected in the regenerated plants (Table 8). From a total of 320 bombarded embryos of four genotypes, Golden Promise, H84012004, Harrington and Phenix, 30 fertile plants were regenerated and these subsequently produced seeds. Twenty-six plants carried the bar gene and 18 expressed a higher tolerance to the glufosinate ammonium. Eleven plants also carried the f gene.

The transformation protocol was very efficient with the tissue culture technique of the invention. Regeneration rates of transgenic fertile plant reached 21% for Golden Promise, 20% for genotype H84012004, 5% for Harrington and 2.5% for Phenix. Success of the co-transformation technique was similar to the expected reporter/marker gene insertion ratio of 50%.

EXAMPLE 7

Biolistic Transformation of Wheat with the bar Gene and a Candidate Yr10 Resistance Gene which Confers Yellow Stripe Rust Resistance

i) Co-transformation

DNA delivery to the tissues was carried out with the Helios Gene Gun System as described in the preceding Examples. The plasmid pCOR113 (Ray Wu, Cornell University) carrying the marker gene, bar, (FIG. 4) and a second vector pCOR113 carrying the candidate Yr10 resistance gene 4B (FIG. 4) were used. An equal mass of these two plasmids (25 μg each) was mixed and coated on 25 mg of 1 μm gold particles and distributed on the inside wall of a 60-cm GoldCoat tubing according to the manufacturer's instructions. Discharge pressure was set at 120–140 psi and a diffusion screen (#165-2475) was used to ensure an even distribution of the gold particles carrying the plasmids.

The tissue culture protocol was essentially the same as that described in Example 2. Wheat scutella were bombarded after two days of culture in DSEM medium. Twenty scutella were transferred onto a modified DSEM medium supplemented with 0.3M mannitol, from 4 h before bombardment to 16 h after bombardment. Forty scutella of the genotype AC Fielder were bombarded with the gold particles. AC Fielder is susceptible to most common races of pathogens causing the stripe rust disease.

ii) Regeneration of Glufosinate-Tolerant Plants

The scutella were transferred to DSEM medium containing 5 mg/L of glufosinate ammonium salt (#C140300, Crescent Chemical, Hauppauge N.Y. USA), 16 h after bombardment.

The further steps of tissue culture were accomplished with this selecting chemical at the same concentration. Embryogenesis was slightly delayed and less frequent during selection procedure using the glufosinate, but there was no impact on the quality of primary and secondary embryos produced.

At the regeneration step, cuttings were done where necessary to isolate tolerant germinating secondary embryos, or groups of secondary embryos, as herbicide sensitive germinating embryos turned yellow. Herbicide-tolerant plantlets were recovered and transferred to potting mix within only two to three months of initiation. In contrast, Becker et al. (1994), report a total time from beginning of culture until the transfer of putative transformed wheat plants to soil of between 15 and 17 weeks.

iii) Characterization of Selected Green Plants

Herbicide-resistant regenerated plants were tested with the leaf brush technique as in the previous Examples. PCR product was probed to confirm presence of the bar DNA in the plant cells. PCR and Southern blot analyses were used to check yellow stripe rust resistance gene insertion. A marker (bar) and the candidate Yr10 resistance gene were detected in regenerated plants. From a total of 40 bombarded scutella, 46 fertile plants were regenerated and produced seeds. Sixty three percent carried the bar gene. However, only eight expressed a very high tolerance to the glufosinate ammonium. Screening for yellow strip rust resistance was very efficient, as all plants but one were susceptible to the pathogen. The resistant plant expressed the characteristic hypersensitive response to the fungal attack. Once again, transformation protocol was very efficient with the tissue culture technique of the invention. The regeneration rate of transgenic plants expressing disease resistance was about 2.5%, which is at least 10 times higher than the expression rate of any heterologous gene in any other protocol currently available.

TABLE 2

Composition of the five tissue culture media used for induction and development of direct somatic embryogenesis of monocots (DSEM), secondary embryogenesis of monocots (SEM), germination of embryos of monocots (GEM) and rapid regeneration of normally developing green and fertile (MS_{reg} and rooting media) cereal plants.[1]

Composition	DSEM medium	SEM medium	GEM medium	MSreg medium	Rooting medium
Salts	mg/L	mg/L	mg/L	mg/L	mg/L
$_{CaCl2}$-$2H_2O$	440.00	440.00	440.00	440.00	440.00
$MgSO_4.7H_2O$	370.00	370.00	370.00	370.00	370.00
NH_4NO_3	165	165.00	165.00	1,650.00	1,650.00
KH_2PO_4	170.00	170.00	170.00	170.00	170.00

TABLE 2-continued

Composition of the five tissue culture media used for induction and development of direct somatic embryogenesis of monocots (DSEM), secondary embryogenesis of monocots (SEM), germination of embryos of monocots (GEM) and rapid regeneration of normally developing green and fertile (MS_{reg} and rooting media) cereal plants.[1]

Composition	DSEM medium	SEM medium	GEM medium	MSreg medium	Rooting medium
KNO_3	1,900.00	1,900.00	1,900.00	1,900.00	1,900.00
$MnSO_4.4H_2O$	22.00	22.00	22.00	22.00	22.00
$CoCl_2.6H_2O$	0.03	0.03	0.03	0.03	0.03
H_3BO_3	6.20	6.20	6.20	6.20	6.20
$ZnSO_4.7H_2O$	8.60	8.60	8.60	8.60	8.60
$CuSO_4.5H_2O$	0.03	0.03	0.03	0.03	0.03
$NaMoO_4.2H_2O$	0.25	0.25	0.25	0.25	0.25
KI				0.83	0.83
$FeSO_4.7H_2O$	27.85	27.85	27.85	27.85	27.85
$NaEDTA.2H_2O$	37.23	37.23	37.23	37.23	37.23
Carbohydrates	mg/L	mg/L	mg/L	mg/L	mg/L
Maltose	15,000.00	15,000.00	15,000.00	15,000.00	
Sucrose	5,000.00	5,000.00	5,000.00	5,000.00	10,000.00
Xylose	350.00	350.00	350.00		
Ribose	350.00	350.00	350.00		
Myo-inositol	200	200	200	250	
Amino acids	mg/L	mg/L	mg/L	mg/L	mg/L
Mix Amino Acid (U2.5)[2]	337.00	337.00	337.00		
L-Glutamine	750.00	750.00	750.00		
Glycine				2.00	
Plant Growth Regulators	μM	μM	μM	μM	μM
Spermine	4.9	4.9	4.9		
Spermidine	27.5	27.5	27.5		
2,4-d	7.9				
PAA	22	11		3.6	3.6
BAP	2.2	111		0.4	
Vitamins	mg/L	mg/L	mg/L	mg/L	mg/L
Pyridoxine.HCl	1.00	1.00	1.00	1.00	
Thiamine.HGl	1.00	1.00	1.00	1.00	
Pantothenate	0.50	0.50	0.50	0.50	
Nicotinic acid	1.00	1.00	1.00	1.00	
Riboflavin	0.20	0.20	0.20	0.20	
Folic acid	0.20	0.20	0.20	0.20	
Biotin	0.20	0.20	0.20	0.20	
Betaine chloride	7.90	7.90	7.90	7.90	
Choline.HCl	10.00	10.00	10.00	10.00	
Ascorbic acid	0.40	0.40	0.40	0.40	
Organic acids	mg/L	mg/L	mg/L	mg/L	mg/L
Malic acid	1,000.00	1,000.00	1,000.00		
Fumaric acid	200.00	200.00	200.00		
Succinic acid	20.00	20.00	20.00		
a-Ketoglutaric acid	20.00	20.00	20.00		
Citric acid	5.00	5.00	5.00		
Pyruvic acid	5.00	5.00	5.00		
Agar purified					6,000.00
Gelrite	3,000.00	3,000.00	3,000.00	3,000.00	
pH	5.80	5.80	5.80	5.80	6.00

[1]Macro-salts and micro-salts composition is identical to MS medium, except for the first three media which have a 10 × reduction in NH4NO3 concentration.

[2]Sigma-Aldrich (Cat. # U-7756). The amino acid composition is L-asparagine, 60 mg/L, arginine, 30 mg/L; g-amino butyric acid, 80 mg/L serine, 55 mg/L; alanine, 30 mg/L; cysteine, 10 mg/L; leucine, 10 mg/L; isoleucine, 10 mg/L; proline, 10 mg/L; lysine, 10 mg/L; phenylalanine, 5 mg/L; tryptophan, 5 mg/L; methionine, 5 mg/L; valine, 5 mg/L; glycine, 2.5 mg/L; histidine, 2.5 mg/L; threonine, 2.5 mg/L.

TABLE 3

Mean numbers (standard deviation) of responding scutella and number of regenerated plantlets originating from germinating primary and secondary embryos.

Barley Genotypes		Number of scutella producing germinating primary embryos per plate (max = 20)	Number of plantlets produced per plate*
H8902001N	6-row forage	18.3 (0.6)	64.7 (10.0)
T89043003NX	6-row forage	8.0 (2.8)	25.5 (4.9)
H84108005NX	6-row forage	16.3 (2.1)	61.0 (5.0)
T89034001	6-row forage	8.0 (1.0)	43.0 (16.4)
Phenix	2-row feed	12.3 (1.5)	53.3 (12.0)
Seebe	2-row feed	12 (1.7)	45.0 (7.5)
Golden Promise	2-row malting	16.3 (1.1)	57.0 (4.0)
Mean (STDEV)		13.0 (4.1)	49.9 (13.4)

*a group of closely germinating embryos was not separated and was further counted as a single plant.

TABLE 4

Mean number of regenerated plantlets and days of culture on media. A plantlet may originate from germinating primary and secondary embryos.

Barley and Wheat Genotypes	replicate *	Days on DSEM	Days on SEM	Number of days to regenerate **	Total plantlets	% ***
AC Nanda	1	6	8	28	157	
(*Triticum aestivum*)	2	6	8–15	28–35	187	
Soft white spring	3	6	8–15	28–35	176	
wheat	4	8	20	42	144	
		6–8	8–20	28–42	664	830
AC Fielder	1	6	6–10	26–36	174	
(*T. aestivum*)	2	6	6–10	26–36	162	
Soft white spring	3	6	9–23	29–42	206	
wheat	4	5	9–23	29–42	157	
		5–6	6–23	26–42	699	874
Golden Promise	1	6–13	12–13	32–40	83	
(*Hordeum vulgare*)	2	9	19	42	163	
2-row malting	3	9	19	42	174	
		6–13	12–19	32–42	420	700
T89034001	1	8	6–29	28–51	101	
(*H vulgare*)	2	8	6–29	28–51	69	
6-row forage	3	8	6–29	28–51	112	
	4	5–10	7–32	26–56	23	
	5	5–10	2–28	21–56	46	
		5–10	2–32	21–56	351	351
H84107004N	1	6	8	28	56	
(*H. vulgare*)	2	6	8–33	28–53	57	
6-row forage	3	6	8–27	28–47	28	
	4	5–11	4–34	23–59	62	
	5	5	6–34	25–53	95	
		5–11	4–34	23–59	298	298
Harrington	1	6–15	7–16	27–45	97	
(*H. vulgare*)	2	5–16	6–16	25–46	30	
2-row malting	3	6	9–16	29–36	41	
	4	6–13	12–19	32–46	100	
		5–16	6–19	25–46	268	335
T89037005X	1	7	6–29	27–50	218	
(*H. vulgare*)	2	7	6–29	27–50	178	
6-row forage	3	5	6–10	25–29	197	
	4	5	6–10	25–29	257	
		5–7	6–29	25–50	850	1062
AC Lacombe	1	7–17	6–29	27–60	197	
(*H. vulgare*)	2	7	6–29	27–50	210	
6-row feed	3	5	6–27	25–46	121	
	4	5–10	7–28	26–52	106	
		5–17	6–29	25–60	634	793
T89047103NX	1	6	6–29	26–49	151	
(*H. vulgare*)	2	6	6–29	26–49	128	

TABLE 4-continued

Mean number of regenerated plantlets and days of culture on media. A plantlet may originate from germinating primary and secondary embryos.

Barley and Wheat Genotypes	replicate *	Days on DSEM	Days on SEM	Number of days to regenerate **	Total plantlets	% ***
6-row feed	3	12	19	45	86	
		6–12	6–29	26–49	365	608

* 20 scutella per plate
** excludes number of days for rooting which was between 3 and 10 days for barley and 0 to 3 for wheat.
*** % of plantlets recovered relative to the original number of scutella cultured.

TABLE 5

Number of regenerated plantlets produced per genotype and percentage of plantlet regeneration per genotype among seven different species of monocots using primary and secondary embryogenesis.

Species	Lines	Number of scutella	Number of regenerated plantlets	Percentage of regenerated plantlets
Triticum aestivum	HY366-BL31	40	754	1885
Triticum aestivum	P8810-B5B3A2A2	20	372	1860
T. durum	DT701	40	150	375
T. monococum	173	60	63	105
T. monococum	238	40	168	420
T. monococum	89	40	159	397
T. urartu	17111	29	49	170
Hordeum vulgare	Golden Promise	40	632	1580
Hordeum vulgare	T89047103NX	40	172	430
Avena sativa	Juniper	30	86	286
Avena sativa	CDC Pacer	20	5	25
Secale cereale	PC Rye	40	6	15

TABLE 6

Number of regenerated green plantlets and percentage of regenerated plantlets from sorghum and corn scutella from immature embryos through direct somatic embryogenesis followed by organogenesis.

	Lines	Rep	Total number of day in culture	Number of regenerated plantlets	Percentage of regenerated plantlets
Sorghum	CK60	1	60	72	360
	CK60	2	60	35	175
	PI229828	1	70	101	505
	PI229828	2	70	74	370
Corn	H96F	1	70	19	95
	H96F	2	70	25	125
	HFDM	1	70	30	150
	HFDM	2	70	22	110

TABLE 7

Characterization of nine individual regenerated barley plants (T_0) transformed with the bar and uidA genes.

		Marker (bar)		Reporter (uidA) Probe	GUS	Southern
#	Genotype	PCR	LBT	PCR	assay	blot
1	Golden Promise	+	s	+	–	·
2	Golden Promise	+	r	–	–	–

TABLE 7-continued

Characterization of nine individual regenerated barley plants (T_0) transformed with the bar and uidA genes.

		Marker (bar)		Reporter (uidA) Probe	GUS	Southern
#	Genotype	PCR	LBT	PCR	assay	blot
5	Golden Promise	+	r	+	–	·
6	Golden Promise	+	r	–	–	·
7	Golden Promise	+	r	+	+	+
8	Golden Promise	+	r	–	–	–
9	Golden Promise	+	r	+	–	·
3	H89108005NX	+	s	–	–	–
4	H89108005NX	+	r	+	–	·

PCR: polymerase chain amplification of either bar or uidA genes using specific primers
LBT: leaf brush technique to verify expression of the bar gene using a small paint brush to apply herbicide (500 mg/L glufosinate ammonium) to a leaf or portion thereof
GUS: b-glucuronidase activity
+: amplification of the specific DNA fragment, hybridization of probe to genomic DNA or positive enzymatic reaction
–: absence of amplification or hybridization or enzymatic activity
·: no data available
r: leaf resistant to herbicide application (no necrosis)
s: leaf susceptible to herbicide application (necrosis)

TABLE 8

Characterization of thirty individual regenerated barley plants (T_0) transformed with the bar and gfp genes.

		Marker (bar)		Reporter (gfp) Probe	Southern
#	Genotype	PCR	LBT	PCR	blot
35	Golden Promise	+	r	–	·
11	Golden Promise	+	r	+	·
12	Golden Promise	+	s	–	–
13	Golden Promise	+	r	–	·
14	Golden Promise	+	r	–	·
15	Golden Promise	+	s	+	+
16	Golden Promise	+	r	+	–
17	Golden Promise	+	r	–	·
18	Golden Promise	+	r	–	·
19	Golden Promise	+	r	–	·
21	Golden Promise	+	s	+	·
22	Golden Promise	–	s	–	·
23	Golden Promise	–	s	–	·
36	Golden Promise	+	r	–	·
37	Golden Promise	–	r	+	+
38	Golden Promise	+	r	–	·
46	Golden Promise	+	r	+	·
57	Golden Promise	+	r	–	·

TABLE 8-continued

Characterization of thirty individual regenerated barley plants (T_0) transformed with the bar and gfp genes.

		Marker (bar)		Reporter (gfp) Probe	Southern
#	Genotype	PCR	LBT	PCR	blot
58	Golden Promise	+	s	–	·
*59	Golden Promise	+	·	–	·
10	H84012004	+	s	+	·
39	H84012004	+	s	–	·
40	H84012004	–	r	–	·
41	H84012004	+	s	–	·
42	H84012004	+	r	+	+
24	H89012004	+	s	–	+
25	H89012004	+	r	+	·
26	H89012004	+	r	–	·
27	Harrington	+	r	+	·
28	Phenix	+	s	+	·

*: plant died
PCR: polymerase chain amplification of either bar or uidA genes using specific primers
LBT: leaf brush technique to verify expression of the bar gene using a small paint brush to apply herbicide (500 mg/L glufosinate ammonium) to a leaf or portion thereof
+: amplification of the specific DNA fragment or hybridization of probe to genomic DNA
–: absence of amplification or hybridization or enzymatic activity
·: no data available
r: leaf resistant to herbicide application (no necrosis)
s: leaf susceptible to herbicide application (necrosis)

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All publications mentioned in this specification are indicative of the level of skill in the art to which this invention pertains. To the extent they are consistent herewith, all publications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. No admission is made that any cited reference constitutes prior art.

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity and understanding it will be understood that certain changes and modifications may be made without departing from the scope or spirit of the invention as defined by the following claims.

What is claimed is:

1. A process for inducing direct somatic embryogenesis in Pooideae and rapidly regenerating fertile plants, comprising the steps of:

a) culturing isolated immature scutella cells of Pooideae in or on a culture medium comprising auxin, cytokinin and polyamine in amounts effective to cause direct formation of primary embryos without an intervening callus stage, at least until at least one primary embryo reaches the globular developmental stage, the auxin being present in greater proportion than the cytokinin; and one of the following steps selected from:

b) culturing one or more of the primary embryos from step (a) under conditions conducive to regeneration of plantlets from the primary embryos, and culturing the primary embryo in or on a regeneration medium; or

c) culturing one or more of the primary embryos at the globular developmental stage and no longer than the coleoptilar stage from step (a) in or on a culture medium comprising auxin, cytokinin, and polyamine in amounts effective to cause induction of secondary embryo formation, at least until secondary embryogenesis is detected, the cytokinin beina present in greater proportion than the auxin, and culturing one or more of the secondary embryos under conditions conducive to regeneration of plantlets from the secondary embryos.

2. The process of claim **1**, wherein, in step (a), the ratio of auxin to cytokinin in the culture medium is from about 5 μM auxin per 1 μM cytokinin to about 20 μM auxin per 1 μM cytokinin.

3. The process of claim **2**, wherein, in step (a) the culture medium includes the plant growth regulators:

i) from about 15 μM auxin to about 45 μM auxin;

ii) from about 15 μM polyamine to about 45 μM polyamine; and

iii) from about 1 μM cytokinin to about 5 μM cytokinin.

4. The process of claim **1**, wherein, in step (a), the ratio of auxin to cytokinin in the culture medium is about 14 μM auxin per 1 μM cytokinin.

5. The process of claim **4**, wherein, in step (a), the culture medium includes the plant growth regulators of:

i) about 30 μM auxin;

ii) about 30 μM polyamine; and

iii) about 2 μM cytokinin.

6. The process of claim 1, wherein, in step (a), the culture medium is DSEM medium.

7. The process of claim 3, wherein steps (a) and (b) are conducted, and the regeneration medium is MS medium.

8. The process of claim 7, further comprising, before step (b), the step of culturing the primary embryo under conditions conducive to germination of the primary embryos until germination of at least one of the primary embryos commences.

9. The process of claim 8, wherein the germination step comprises culturing the primary embryo in or on a culture medium which comprises polyamine in an amount effective to cause germination of the primary embryos, and which is essentially free of either auxin or cytokinin.

10. The process of claim 9, wherein the culture medium comprises from about 15 μM polyamine to about 45 μM polyamine.

11. The process of claim 9, wherein the culture medium comprises about 30 μM polyamine.

12. The process of claim 9, wherein the germination step comprises culturing the primary embryo in or on GEM medium.

13. The process of claim 7, further comprising the step of culturing the plantlets under conditions conducive to induction of root formation until the plantlets form roots.

14. The process of claim 13, further comprising the step of transplanting the plantlets to soil and growing them to maturity.

15. The process of claim 14, wherein the scutella cells are selected from the genera consisting of *Triticum, Hordeum, Secale, Avena, Dactylis*, and *Bromus*.

16. The process of claim 15, wherein the scutella cells are selected from the group consisting of *Triticum durum* amphiploids, *Hordeum vulgare, Triticum aestivum, Triticum durum, Triticum monococum, Triticum urartu, Secale cereale*, and *Avena sativa* scutella cells.

17. The process of claim 15, wherein the scutella cells of step (a) are free of a germ.

18. The process of claim 17, which further includes, after step (a), cutting the scutellum carrying the primary embryo into a plurality of pieces prior to culturing in step (b).

19. The process of claim 18, wherein the scutellum carrying the primary embryo is cut into two to four pieces.

20. The process of claim 15, wherein step (a) further comprises the step of introducing foreign DNA into the scutella cells or primary embryo so that the foreign DNA becomes stably integrated into the genome of the cells.

21. The process of claim 20, wherein the foreign DNA is introduced into the scutella cells or primary embryo by particle bombardment or by *Agrobacterium*-mediated transformation.

22. The process of claim 21, wherein the foreign DNA is introduced into the scutella cells or primary embryo in step (a) during the development of the primary embryo.

23. The process of claim 22, wherein the foreign DNA is introduced into the scutella cells between zero to five days after commencement of tissue culture.

24. The process of claim 22, wherein the foreign DNA is introduced into the scutella cells or the primary embryo after two days following commencement of tissue culture.

25. The process of claim 22, wherein after the foreign DNA has been introduced, the scutella cells or primary embryo are transferred to a media for steps (a) and (b) which includes a selective agent to identify a transformed plant cell that has incorporated the foreign DNA.

26. The process of claim 25, wherein the transformed plant cell is cultured in media to support regeneration of transformants.

27. The process of claim 26, which further comprises confirming expression of the foreign DNA in the transformants by one or both of polymerase chain reaction and Southern blot analyses.

28. The process of claim 3, wherein, in step (c), the ratio of auxin to cytokinin in the culture medium is from about 0.05 μM auxin per 1 μM cytokinin to about 0.2 μM auxin per 1 μM cytokinin.

29. The process of claim 28, wherein, in step (c), the culture medium includes the plant growth regulators:

i) from about 5 μM auxin to about 15 μM auxin;

ii) from about 15 μM polyamine to about 45 μM polyamine; and

iii) from about 50 μM cytokinin to about 200 μM cytokinin.

30. The process of claim 3, wherein, in step (c) the ratio of auxin to cytokinin is about 0.1 μM auxin per 1.0 μM cytokinin.

31. The process of claim 30, wherein, in step (c), the culture medium includes the plant growth regulators of:

i) about 11 μM auxin;

ii) about 30 μM polyamine; and

iii) about 110 μM cytokinin.

32. The process of claim 3, wherein, in step (c), the culture medium is SEM medium.

33. The process of claim 32, wherein step (c) comprises culturing the secondary embryo in or on a regeneration medium.

34. The process of claim 33, wherein the regeneration medium is MS medium.

35. The process of claim 33, further comprising, before step (c), the step of culturing the secondary embryo under conditions conducive to germination of the secondary embryos until germination of at least one of the secondary embryos commences.

36. The process of claim 35, wherein the germination step comprises culturing the secondary embryo in or on a culture medium which comprises polyamine in an amount effective to cause germination of the secondary embryos, and which is essentially free of either auxin or cytokinin.

37. The process of claim 36, wherein the culture medium comprises from about 15 μM polyamine to about 45 μM polyamine.

38. The process of claim 36, wherein the culture medium comprises about 30 μM polyamine.

39. The process of claim 36, wherein the germination step comprises culturing the secondary embryo in or on GEM medium.

40. The process of claim 33, further comprising the step of culturing the plantlets under conditions conducive to induction of root formation until the plantlets form roots.

41. The process of claim 40, further comprising the step of transplanting the plantlets to soil and growing them to maturity.

42. The process of claim 41, wherein the scutella cells are selected from the genera consisting of *Triticum, Hordeum, Secale, Avena, Dactylis*, and *Bromus*.

43. The process of claim 42, wherein the scutella cells are selected from the group consisting of *Triticum durum* amphiploids, *Hordeum vulgare, Triticum aestivum, Triticum durum, Triticum monococum, Triticum urartu, Secale cereale*, and *Avena sativa* scutella cells.

44. The process of claim 42, wherein the scutella cells of step (a) are free of a germ.

45. The process of claim 44, which further includes, after step (a), cutting the primary embryo into a plurality of pieces before culturing in step (c).

46. The process of claim 45, wherein the primary embryo is cut into two to four pieces.

47. The process of claim 45, which further comprises, before step (c), the step of cutting the primary embryo carrying the secondary embryo into a plurality of pieces, or cutting a germinating leaf if developed, to obtain a high frequency of germination of secondary embryo.

48. The process of claim 47, wherein the primary embryos carrying the secondary embryo is cut into two pieces.

49. The process of claim 42, wherein step (a) further comprises the step of introducing foreign DNA into the scutella cells or the primary embryo so that the foreign DNA becomes stably integrated into the genome of the cells.

50. The process of claim 49, wherein the foreign DNA is introduced into the scutella cells or primary embryo by particle bombardment or by *Agrobacterium*-mediated transformation.

51. The process of claim 50, wherein the foreign DNA is introduced into the scutella cells or the primary embryo in step (a) during the development of the primary embryo.

52. The process of claim 51, wherein the foreign DNA is introduced into the scutella cells or the primary embryo between zero to five days after commencement of tissue culture.

53. The process of claim 51, wherein the foreign DNA is introduced into the scutella cells or the primary embryo after two days following commencement of tissue culture.

54. The process of claim 51, wherein after the foreign DNA has been introduced, the scutella cells or primary embryo are transferred to a media for step (c), and optionally for step (a), which includes a selective agent to identify a transformed plant cell that has incorporated the foreign DNA.

55. The process of claim 54, wherein the transformed plant cell is cultured in media to support regeneration of transformants.

56. The process of claim 55, which further comprises confirming expression of the foreign DNA in the transformants by one or both of polymerase chain reaction and Southern blot analyses.

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